INTERACTION OF CHEMICAL MUTAGENS WITH DNA
(USE OF HYDROXYAPATITE CHROMATOGRAPHY AND S1 NUCLEASE)

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SUMMARY AND CONCLUSION

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These studies were aimed at investigating the changes in secondary structure of DNA due to the binding of various mutagenic drugs. Some representative intercalating and non intercalating dyes and drugs were chosen. In addition, the effect of the ubiquitous vitamin riboflavin and mutagenic flavonoid quercetin on DNA was also studied. Certain techniques and tools such as hydroxyapatite chromatography, BND-cellulose chromatography and single strand specific S_1 nuclease were used. The usefulness of these methods in nucleic acid studies is already well documented. Our studies are therefore, an extension of these methods in elucidating the structure of various DNA-drug complexes.

The binding properties of DNA complexed with intercalating agents (ethidium bromide, acriflavine, actinomycin D and acridine orange) to hydroxyapatite (HA) were found to be quite different than of native DNA alone. DNA was eluted from HA columns complexed with the intercalating agent and at a significantly lower phosphate molarity than untreated DNA. With non-intercalating agents (spermine and berenil) no such alteration in the elution pattern was observed and DNA and the ligand eluted separately. When ethidium bromide was extracted from chromatographed DNA and the DNA rechromatographed on hydroxyapatite, it again behaved as double stranded material. The degree of binding of ethidium bromide to DNA was

quantitated on HA and was found to be comparable to the Scatchard plot analysis of the spectrophotometric data of the binding of the dye to DNA. These investigations provide an additional method, which is simple and fairly rapid, of determining the mode of binding to DNA of polycyclic aromatic and other ligands.

During the course of these studies a new and sensitive method for the colorimetric estimation of the trypanocidal drug, berenil was also developed and its binding in vitro to hemoglobin and other serum fractions established.

S_1 nuclease hydrolysis and benzoylated naphthoylated DEAE cellulose (BND cellulose) chromatography was used to demonstrate that alkylation of DNA by dimethyl sulfate at neutral pH leads to the production of partially denatured molecules under conditions where no significant depurination occurs. DNA was alkylated with increasing concentrations of the alkylating agent and subjected to enzymatic degradation and binding to BND cellulose. An increasing degree of DNA hydrolysis and adherence to BND cellulose was seen. On hydroxyapatite chromatography the alkylated DNA still eluted at the position of double stranded molecules suggesting the presence of partially denatured regions. The presence of salt had a preventive effect on such denaturation.

S_1 nuclease was also used to explore the changes in the secondary structure of DNA complexed with intercalating and

non intercalating agents. A progressive decrease in the rate and extent of hydrolysis of DNA complexed with increasing relative concentrations of the known intercalator, ethidium bromide was observed. With increasing enzyme concentration, the decrease in maximum DNA hydrolyzed was considerably greater in the case of ethidium bromide than with spermine, a non intercalating agent. S_1 nuclease was also used to determine the thermal melting profile of native DNA complexed with increasing molar ratios of ethidium bromide and acridine orange. The melting profile was found to be biphasic with both the drugs. Also, complete hydrolysis of DNA was not observed even at 95°C and progressively decreasing maximum hydrolysis was seen. These observations are in agreement with the property of intercalating agents of stabilizing the secondary structure of DNA. However, such biphasic melting profiles were characteristically absent in case of DNA treated with non intercalating agents like streptomycin sulfate and spermine. These results are possibly accounted for, by the property of intercalating agents of stabilizing the secondary structure of DNA and their reported preference in binding to G-C base pairs.

Dimethylsulfoxide and formamide are two solvents which destabilize the secondary structures in nucleic acids. The usefulness of S_1 nuclease can be enhanced if it can be shown to be active in these solvents. S_1 nuclease exhibited no inhibition of activity with alkylated DNA in presence of 5%

dimethyl sulfoxide and formamide. Presence of increasing concentration of sodium chloride affected the hydrolysis of denatured, alkylated and depurinated DNA by S_1 nuclease to about the same degree. Treatment of depurinated DNAs prepared at 40°C, 50°C, 60°C and 70°C at pH 3.5 with S_1 nuclease exhibited an increasing degree of maximum hydrolysis which was consistent with the fact that higher temperature leads to increased depurination causing increasing separation of the two strands.

S_1 nuclease hydrolysis and BND cellulose chromatography were employed to study the effect of riboflavin and visible light on DNA. Native calf thymus DNA was incubated with riboflavin in the presence of fluorescent light for various time periods (4 hours and 40 hours respectively) and subjected to S_1 nuclease hydrolysis. An increasing degree of DNA degradation was seen suggesting a destabilization of the secondary structure. A decrease in melting temperature was also observed. Incubation with riboflavin and illumination caused adherence to BND cellulose indicating the production of single stranded regions or breaks in the native double stranded molecules. However, when incubation was done in dark and in the presence of triplet excited state quencher, potassium iodide, a reduced adherence of DNA to BND cellulose was seen. Plasmid pBR322 DNA was also treated with riboflavin under these conditions and subjected to agarose gel electrophoresis. No degradation could be seen in dark incubated and potassium iodide

treated samples. These results indicate that the adherence of DNA to BND cellulose in dark is possibly due to the binding of aromatic residues to the resin suggesting the formation of a complex between riboflavin and DNA.

In vitro experiments were also conducted to study the DNA modifying effects of the mutagenic flavonoid quercetin. The rate of DNA hydrolyzed after 4 hours of pretreatment with quercetin was found to be only about 50% of that in its absence. However, after 10 and 24 hours of preincubation with the drug, the rate of S_1 nuclease hydrolysis of treated DNA was found to be greater than that of native DNA. Thermal melting profiles of DNA preincubated for 10 and 24 hours respectively indicate a slight decrease in melting temperatures ($2^{\circ}C$ and $4^{\circ}C$ respectively). These results suggested a destabilisation of the secondary structure, either through the creation of partial single stranded regions or single stranded breaks in DNA. Sepharose 6B chromatography of native DNA which had been digested with S_1 nuclease after preincubation with quercetin for 24 hours indicate the production of various sized degraded molecules, a major part of which was equivalent in size to the digestion products of heat denatured DNA. Digestion of quercetin treated and untreated DNA by a mixture of DNase I and S_1 nuclease showed that after 10 minutes of enzyme treatment only 40% of quercetin treated DNA is hydrolyzed whereas the hydrolysis of untreated DNA is more than 80%.

These results indicate that the initial interaction of quercetin with DNA may have a stabilizing effect on its secondary structure but prolonged treatment leads to an extensive disruption of the double helix.

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Date : Sept. 21, 1983

CERTIFICATE

I certify that the work presented in this thesis has been carried out by Mr. Nasir Kamal Alvi under my supervision. It has not been submitted for any other degree and unless otherwise stated is all original.

(S.M. HADI)
Reader in Biochemistry
DIVISION OF BIOCHEMISTRY

CONSECRATED
TO
THE REVERED MEMORY OF
MY MOTHER
WHOSE BENEDICTIONS WILL ALWAYS REMAIN
THE GUIDING LIGHT OF MY CAREER

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

DNA INTERCALATING AGENTS

The elucidation of the structure of biological macromolecules at the primary, secondary and tertiary levels is essential to an understanding of biological function. This is a fundamental principle underlying the success of modern molecular biology. There is increasing evidence that covalent binding of chemical carcinogens to cellular macromolecules, particularly nucleic acids, is the initial critical event in the encounter between an environmental carcinogen and target cells in the exposed host (Miller, 1970; Weinstein, 1977). Therefore, to understand carcinogenesis at a molecular level, it becomes necessary to understand the complete chemical structure and stereochemistry of the carcinogen-macromolecular adducts, to then determine the associated conformational changes in the target macromolecules, and finally to relate these chemical and physical findings to possible aberrations in the functioning of the chemically modified macromolecules. Polycyclic aromatic hydrocarbons such as benzo (a) pyrene, are ubiquitous in the environment and are of increasing concern as carcinogens. Several physical studies have shown that binding of polycyclic aromatic ligands to DNA can occur through at least two distinct modes.The binding mode of lowest free energy has been interpreted as intercalation (Lerman, 1964; Peacocke and Skerett, 1956; Muller and Crothers, 1968; Fiel et al., 1979). This is characterized by the

interaction of the nucleotide bases with the planar ligand. The weaker binding mode is generally described as an electrostatic externally bound ligand, and is therefore characterized by a predominant interaction of the ligand molecules stacked along the external surface of the DNA.

X-ray crystallographic studies have provided direct evidence for intercalation (Tsai et al., 1977; Sakore et al., 1977). However, several other methods also have provided support for this model. For example, an enhanced stability of the intercalation complex has been noted by an increase in Tm (Lerman, 1964). Viscosity and sedimentation measurements apparently reflect an increase in the chain length of DNA, which results from the insertion of the ligand (Cohen and Eisenberg, 1969). Circular dichroism studies have shown the existence of multimode binding to DNA, and measurements of very high affinity found for several planar ligands to DNA have all been interpreted as intercalation.

Intercalation was first proposed by Lerman (1964) as a model to explain the interaction between acridines and DNA (Peacocke and Skerett, 1956). It leads naturally to plausible hypotheses for the mechanism by which acridines produce frame shift mutations (Brenner et al., 1961; Lerman, 1963; Streisinger, 1966). However, during the last two decades since the model was originally conceived it has become apparent that the phenomenon of intercalation may extend to a much wider range of drugs than just the acridines. Among these are drugs variously

important as chemotherapeutic agents, carcinogens, tranquilizers, hallucinogens and biochemical tools. Examples, for all of which intercalation has been specifically suggested as the mode of interaction with DNA include the trypanocide ethidium bromide, the antitumor antibiotics daunomycin and nogalamycin, the schistosomicide miracil D, the antimalarial drug chloroquine, the carcinogen 3,4-benzpyrene, the radical ion of the tranquilizer chloropromazine, the hallucinogen lysergic acid diethylamide (LSD) and actinomycin D. Clearly, if all these drugs do bind to DNA by intercalation, and yet that binding leads ultimately to such diverse biological manifestations, the intercalation process must provide a strikingly versatile molecular basis for drug action.

Particularly important as diagnostic feature of the intercalation process are two related effects on the hydrodynamic behaviour of DNA; an increase in viscosity and decrease in sedimentation coefficient (Kersten, 1966; O'Brien, 1966; Muller and Crothers, 1968) of covalently closed superhelical DNA. These effects occur because the helix plus intercalated drug behaves as a stiffer, more slender rod in solution. However, intercalation also requires that the helix become partially uncoiled at the point of insertion of a drug molecule between the base pairs (Fuller and Waring, 1964). The need for this local uncoiling arises because the sugar - phosphate chain is already fairly well extended in normal B form DNA (Fuller and Waring, 1964).

The uncoiling associated with an intercalation reaction has been evidenced by studies on closed circular duplex DNAs (Crawford and Waring, 1967; Bauer and Vinograd, 1968). Because of the topological peculiarities inherent in their circularity such DNAs display large changes in their supercoiling in response to the local uncoiling caused by intercalation. During the first phase of drug binding the number of right handed supercoils originally present in the circles decreases. At a critical level of binding the supercoils are just removed so that the DNA molecules behave as untwisted open circles; at this point the accumulated uncoiling due to intercalation has, in effect, titrated the initial deficiency of turns in the circular double helix which gave the circles the original right handed supercoils. As further drug binding takes place, the accumulated uncoiling causes the appearance of left handed supercoils. Since supercoiled circles are more compact than the equivalent open circles, they sediment more rapidly, so the loss and reappearance of supercoils are easily observed by the changes in sedimentation coefficient.

The intercalating dye ethidium bromide has now become a valuable tool for measuring the number of supercoils in closed circular duplex DNAs (Wang, 1969a; Wang, 1969b; Waring, 1970). Waring (1970) has further tested the effect of 17 substances on the $S_{20}W$ of ϕX174 RF DNA with the object of investigating whether changes in the supercoiling of closed circles may be

employed to verify intercalative binding. Proflavine, hycanthone, daunomycin, nogalamycin, chloroquine, propidium and actinomycin D, all affect the supercoils in the same qualitative fashion as ethidium. Non intercalating substances tested include spermine, streptomycin, berenil, chromomycin and methramycin; none appeared to remove and reverse the supercoiling.

HYDROXYAPATITE CHROMATOGRAPHY OF NUCLEIC ACIDS

Hydroxyapatite (HA) was originally developed as a medium for the fractionation of proteins (Tiselius et al., 1956; Hjerten, 1959). It was later used by Semenza (1957) and Main et al. (Main and Cole, 1957; Main et al., 1959a; Main et al. (1959b) for the chromatography of nucleic acids. However, Bernardi carried out the most extensive investigations on the chromatography of nucleic acids on HA columns (Bernardi, 1962). He recognized that HA can discriminate nucleic acids endowed with different secondary structures; rigid ordered structures having more affinity for HA than flexible disordered ones. The simplest use to which HA chromatography can be put is to separate native double helical DNA from denatured DNA. However, subtle differences in the secondary and tertiary structures of nucleic acids can also be discriminated by HA columns (Bernardi, 1965; Bernardi, 1969; Bernardi et al., 1968; Bernardi et al., 1970), since they give rise to different distributions of groups

available for the interaction with the adsorbing sites on HA. Thus it is possible to separate glucosylated DNA of T-even phages from non glucosylated DNA, superhelical circular DNA from open linear form and mitochondrial yeast DNA from nuclear yeast DNA(Bernardi, 1971). Double stranded RNA behaves like native DNA and therefore can be separated from single stranded RNA. An excellent idea of the discriminating power of HA columns with respect to the secondary and tertiary structures can be had from the fact that remarkable separations of tRNAs of various amino acids have been obtained(Pearson and Kelmers, 1966).

The main factor involved, as suggested by Bernardi (1971) in the adsorption of nucleic acids on HA seems to be the interaction between the phosphate groups of nucleic acids and Ca^{++} on the surface of HA crystals. This has been suggested by several observations such as treatment of HA with compounds having a strong affinity for Ca^{++} e.g. EDTA, polyphosphates etc. These compounds decrease the adsorption capacity of HA for nucleic acids. Elution of polynucleotides from HA by phosphates is presumably due to a specific competition between PO_4^{---} of the eluent and phosphate groups of polynucleotides for adsorbing sites on HA, and not simply due to an increase in ionic strength. According to Bernardi (1971) elution can be performed at a practically constant ionic strength; native DNA being eluted at the same phosphate molarity independently of the ionic

strength of the eluting buffer.

The property of HA columns of discriminating ordered, rigid structures from disordered flexible ones is quite general. An explanation for this phenomenon is that phosphate and carboxyl groups which were available for the interactions with adsorbing sites on HA in the rigid, ordered structures greatly decrease in number on the outer surface of the randomly coiled, denatured nucleic acids. A similar explanation has been suggested by Bernardi for the decreased affinity for HA observed for the twisted circular forms of polyoma virus DNA (Bourgaux, et al., 1967). Furthermore, local concentration of phosphate or carboxyl groups due to the existence of rigid secondary and tertiary structures disappear upon denaturation. An example of the importance of charge distribution in the chromatography on HA columns is given by the strikingly different behaviour of nucleoside triphosphates and trinucleotides which have the same net charge; nucleoside triphosphates are eluted by 0.2M potassium phosphate buffer whereas trinucleotides are eluted by 0.001M potassium phosphate buffer.

Lately HA chromatography has been used to isolate closed circular duplex DNA molecules and to separate mixtures of DNA of varying G + C content in the presence of the intercalating agent ethidium bromide and base specific complexing agents. Pakroppa et al. (1975) have described a method, using chromatography on HA in the presence of ethidium bromide, for the

preparative purification of supercoiled DNA of bacterial cell extracts. The method is based on the differential binding ability of the dye for linear and closed circular DNA. The extent of separation seems to depend only on the intrinsic super helical density of the supercoiled DNA molecules and not on their sizes. Pakroppa and Muller (1974) have also described a HA chromatographic technique for separating mixtures of DNA of varying G + C contents. The method employs a G + C specific DNA ligand (2-methyl - 3 amino - 7-dimethylamino-5-phenyl-phenazinium cation). DNA molecules rich in G + C pairs bind larger amounts of this dye and are eluted earlier from the HA column than are molecules rich in A+T pairs. Elution of the DNA is not dependent on molecular weight, so samples of different molecular weight can be separated on the basis of G + C content. The technique should be specially useful for separating G + C rich minor components from DNA obtained from eukaryotic cells. Colman et al. (1978) have recently published a rapid purification procedure for the purification of plasmid DNAs by HA. This method involves chromatography, at room temperature, of bacterial cleared lysates in the presence of high concentrations of phosphate and urea. The method is extremely rapid and amenable to large scale plasmid preparation and has become a standard procedure for the purification of various plasmid vectors used in recombinant DNA research.

Extensive work carried out by Bernardi (1971) and Martinson (1973a, 1973b) has firmly established that increases in structural order are correlated with increases in affinity for HA. However, in the case of separation of double stranded molecules the situation is slightly complex. Martinson (1973c) has postulated that the affinity of a rigid, helical nucleic acid for HA is governed by the steric availability of its backbone phosphate for adsorption interactions. He found that dsRNA and DNA-RNA hybrid have less affinity for HA than dsDNA. This observation has been explained on the basis that phosphate groups of DNA protrude from the helix and are apparently relatively accessible for surface interaction while the phosphate groups of both ds-RNA and DNA-RNA hybrid appear buried in the surface of the helix. Martinson has also shown that ds-DNA is fractionated on HA according to base composition; DNA rich in G+C has slightly less affinity for HA than DNA rich in A+T. HA chromatography of short ss-DNA has been studied by Wittelsberger and Hansen (1979). The results indicated that single stranded fragments below 50 nucleotides elute from HA appreciably before high molecular weight denatured DNA using phosphate gradients. This is an important consideration when using HA to fractionate DNA populations which contain short strands.

S_1 NUCLEASE AND ITS USE AS A TOOL IN PROBING THE STRUCTURE OF NUCLEIC ACEDS.

S_1 nuclease is a member of a class of nucleases, widely distributed among fungi and higher plants, which possess a pronounced specificity towards nucleic acid substrates lacking ordered structures. These enzymes are also called "single strand specific nucleases". It has been isolated and well characterized from Aspergillus oryzae by several investigators (Ando, 1966; Sutton, 1971; Vogt, 1973; Rushizky et al., 1975; Oleson and Sasakuma, 1980). It is a heat stable glycoprotein as evidenced by its affinity for Con A-sepharose and its elution from the immobilized lectin by methyl - -D mannoside and requires Zn^{++} for maximal activity. S_1 nuclease selectively hydrolyzes single stranded DNA, RNA, 3'AMP and 2' AMP in acidic medium. The relative rates of hydrolysis at pH 4.5 are ssDNA RNA 3'AMP 2'AMP. Three forms of the enzymes with differing isoelectric point (pI) are observed on electrofocussing and each form exhibits the same relative activity on single stranded DNA and 3'AMP. S_1 nuclease exhibits activity over a broad range of pH with maximal activity at pH 4.3-4.6. Ribonucleotides are hydrolyzed 100 fold more rapidly than deoxyribonucleotides. Evidence indicates that the 2' and 3' nucleotidase activities are associated with S_1 nuclease.Nearly homogeneous preparations of S_1 nuclease are unable to catalyze the hydrolysis of 5' AMP but they exhibit hydrolytic activity

towards 3' AMP and to a lesser degree towards 2' AMP. The 3' nucleotidase activity of the enzyme is inhibited by ssDNA but not dsDNA. S_1 nuclease hydrolyzes ribonucleoside 3' phosphates at a high rate but is much less active on 2' AMP and deoxyribonucleoside-3'-phosphates. The efficient hydrolysis of phosphomonoester bond in mononucleotide substrates is found to occur over a broad pH range in contrast to that of ssDNA. Maximal activity with 3' AMP and adenosine 3' 5' -biphosphate is observed at pH 6-7. S_1 nuclease exhibits maximal activity in 0.01M NaCl and with greater concentrations a gradually increasing inhibition is observed. Both hydrolysis of polynucleotides and 3' AMP are stimulated in presence of Zn^{++}. Mercaptoethanol provides substantial protection to the enzyme during exposure to low pH.

The specificity of S_1 nuclease from Aspergillus oryzae has been extensively studied by Weigand et al. (1975). They have described conditions for digesting single stranded DNA by S_1 nuclease without introducing breaks in double stranded DNA. The enzyme was inhibited by low concentrations of phosphate compounds. 50% inhibition is caused by 2 mM phosphate or 20 uM AMP and 1 uM dATP. The enzyme is highly specific for ssDNA and the rate of breakage of phosphodiester bonds in ssDNA is atleast 75,000 times greater than that in dsDNA. S_1 nuclease makes many single strand breaks in ultraviolet-irradiated duplex DNA. Cleavage of ultraviolet-irradiated

DNA has also been observed by Shishido and Ando (1974). In one of its strands the bacteriophage T5 DNA contains nicks at genetically determined loci (Abelson and Thomas, 1966). Each interruption is a scission of only a single phosphodiester bond which can be closed by polynucleotide ligase. After digestion T5 DNA (Mol. Wt. 75 million) by S_1 and comparison of $S_{20}W$ of various species formed in a neutral sucrose gradient it is found that the pattern of cleavage is consistent with that expected as if S_1 were cutting the strand opposite the nicks in T5 DNA. Cleavage of the strand opposite a nick has been inferred also from the studies of S_1 cleavage of superhelical DNA. It has been shown that the superhelical DNA of ϕx 174 (Form I) is converted first to a relaxed circular molecule (Form II), and then to a linear molecule (Form III) by cleavage at one site per molecule on treatment with S_1 nuclease. Cleavage products of S_1 nuclease action on ϕx 174 DNA can be separated by sucrose gradient density centrifugation (Weigand et al., 1975). Godson (1973) also reported that ϕx 174 RFI DNA is cleaved by S_1 nuclease. Beard et al. (1973) and Mechali et al. (1973) described the cleavage of superhelical SV40 DNA by S_1 nuclease. Germond et al. (1974) found that S_1 nuclease cleaved superhelical polyoma DNA. In all these cases the cleavage appeared to be limited largely to one or two cuts necessary to convert superhelical DNA to a relaxed circular form or to a linear form. This limited action suggested that the cleavage was a consequence

of some peculiarity of superhelical DNA. In case of SV40 DNA, the limited cleavage occurred within one or two small regions (Beard et al., 1973). A substrate is also formed by a hetero-duplex DNA molecule in which part of an unpaired loop contains two possible sites of action of S_1 nuclease; the loop itself and the site on the other strand which is opposite the loop. It has been demonstrated that under certain conditions S_1 nuclease cleaves a loop in one strand of heteroduplex.DNA while leaving the opposite strand intact (Weigand et al., 1975). In order to determine whether S_1 nuclease cleaves at a few specific sites or a large number of sites, the effect of S_1 digestion of ø: 174 RFI DNA on the pattern of fragments produced by restriction endonucleases was studied. The products of digestion of øx 174 RFI DNA by S_1 nuclease were degraded completely to fragments by endonuclease R. Hind (Smith and Wilcox, 1970) or endonuclease R. Hae (Middleton et al.,.1972) and the patterns of fragmentation were observed by gel electrophoresis and autoradiography. It was concluded that cleavage of S_1 nuclease occured at more than a few specific sites and that the sites of cleavage of øx 174 RFI DNA by S_1 nuclease are distributed throughout the molecule, although the distribution is not random. Similarly, when digested by S_1 nuclease, the superhelical replicative form (RFI) DNA of icosahedral phages G4 and G14 (Godson, 1974) was also converted rapidly to form II and more slowly to Form III. Further, studies demonstrated that the superhelical

DNAs of phages G4 and G14 were not cleaved by S_1 nuclease at a unique or even at a few strongly preferred sites. This further strengthens the inference that the sites of cleavage are probably distributed about the molecules as was the case with ϕx 174 RFI DNA (Weigand et al., 1975).

Two distinct disadvantages of using S_1 nuclease as a tool are its requirements of divalent cations and the optimum pH range which is sufficiently acidic to cause appreciable depurination of DNA, especially with extended digest times (Vogt, 1973). However, extraneous nicking due to acid depurination can be reduced sufficiently for many purposes by using concentrations of salt and hydrogen ion that are different from those at which the enzyme acts most rapidly, viz., 0.1M NaCl instead of 0.01M and pH 5.0 instead of pH 4.5. S_1 nuclease also displays anamolous reaction kinetics at low substrate concentrations.

The activity of S_1 nuclease in solutions which destabilize DNA secondary structure has been investigated by Case and Baker (1975). It has been demonstrated that the enzyme retains varying amounts of single stranded DNA specific nuclease activity in solvents which perturb or destabilize the secondary structure of DNA, viz., formalhedyde, formamide and glyoxal. S_1 nuclease works efficiently at HCHO concentrations upto or less than 5% but its activity is sufficiently reduced at higher concentration (10-20%). Thus an ideal formaldehyde concentration for optimum

S_1 nuclease activity would be that which does not allow subsequent denaturation of base pairs adjacent to formylated bases in the destabilized regions at a temperature at which S_1 nuclease digestion will efficiently take place. A formamide concentration of 2.5 - 10 % does not affect the ability of S_1 nuclease to hydrolyze heat denatured DNA. However, at 20% formamide concentration significant decline in the rate of digestion of substrate is observed which is drastically reduced at 30% and is almost zero at 40% or 50% formamide. Addition of formamide reduces the mid range melting temperature (Tm) of DNA by 0.6-0.7°C for each percent of formamide added (Bluthmann et al., 1973; and McConaughy et al., 1969). The Tm of both sea urchin and ø80 phage DNA melted in 50% formamide was reduced by approximately 31°C compared to the control without formamide. Finally glyoxal tested at concentration of 0.25M or less does not affect the ssDNA specific activity of S_1 nuclease. Glyoxal can form a stable adduct with non H-bonded guanine residues analogous to the reaction of formaldehyde with cytosine and guanine. Therefore, glyoxal could presumably fix guanine and cytosine rich sequences contained within large destabilized adenine and thymine rich region of a partially denatured DNA molecule. The activity of S_1 nuclease in denaturing solvents has also been probed by Hutton and Wetmar (1975). They have reported activity in aqueous solutions of greater than 60% formamide, 50% dimethylsulfoxide, 30% dimethylformamide and 2% formaldehyde. The single strand specificity of S_1 nuclease

is retained in 50% formamide at 37°C, 3°C below the DNA melting temperature. S_1 is shown to be active at temperatures greater than 5°C above the DNA melting temperature in this solvent. Their results are contrary to those reported by Case and Baker (1975) in which they found S_1 nuclease activity much reduced in 30% and completely absent in 40% formamide solutions.

These studies have led to the use of S_1 nuclease as a probe of renaturation kinetics (Sutton, 1971). The enzyme has been found to be capable of removing the single stranded regions from the reassociated DNA molecules at a range of temperatures without extensively degrading the reassociated duplex regions. S_1 nuclease has been used for investigating variations in the secondary structure of DNA (Shishido and Ando, 1975) and as a reagent for mapping splice points (Berk and Sharp, 1978) in RNA molecules. Wurst et al. (1978) have described a rapid general method for mapping the structure of RNA using S_1 nuclease as a probe. The method has the potential to determine the exact locations of paired and unpaired bases in the sequences and to detect conformational transitions induced by changes in the physical state of the solution. The data presented by Wurst et al. (1978) demonstrate that full length transfer RNAs namely $tRNA^{Phe}$ and $tRNA^{Glu}_2$ have limited susceptibilities to S_1 nuclease. However, S_1 nuclease under conditions designed to generate partial digestion attacks these molecules in their loops,

with particular preference for the anticodon loops. S_1 nuclease also hydrolyzes, though to a limited extent, at particular sites in the D and T loops in these molecules. Thus the RNA structure mapping approach can be successfully exploited for generating detailed information concerning the locations of hairpin single stranded loop and helical stem regions in $5'^{32}P$ labelled RNA molecules.

Case et al. (1974) have examined long term sea urchin embryo DNAs for size of recovered pieces, single strandedness and length of continuous double stranded regions using S_1 nuclease. It was concluded that the decrease in size of the sea urchin DNA with S_1 nuclease treatment as evidenced by the sedimentation data obtained from neutral sucrose gradient or by the amount of DNA digested by S_1 nuclease is a result of the removal of single stranded regions. These data have further demonstrated the existence of single stranded or gapped regions in native sea urchin DNA. Godson (1973) has reported that S_1 nuclease introduces single strand breaks in double stranded, supercoiled ϕx174 RFI, double stranded relaxed ϕx 174 nicked circular RF II DNA and linear double stranded T-7 DNA. The double stranded DNA nicking activity of S_1 nuclease is low compared with the single stranded DNA digestion activity. A technique emphasizing the utility of single strand specific nucleases for the single strand interruptions in DNA was used by Meneghini (1976) using purified DNA and the single strand

specific endonuclease from Neurospora crassa for the detection of interruptions in newly synthesized DNA. S_1 nuclease has been used by Sheridan and Huang (1977) to measure repair by determining the resistance to alkaline denaturation of DNA with varying number of breaks. Karran et al. (1977) have used S_1 nuclease for the detection of discontinuities in cellular DNA caused by the treatment of human lymphoid cells with methylmethanesulfonate (MMS) and acetoxyacetyl aminoflourene (AAAF). In their studies, the investigators applied S_1 nuclease to lysates on the top of a gradient for the analysis of single strand interruptions induced by chemical carcinogens. In combination with the Mcgrath Williams technique (1966) this enzyme treatment approach of Karran et al. (1977) is a useful analytical tool for exposing single strand breaks. Besides these an insight into the varied usage of S_1 nuclease can be had from the following examples (a) the quantitation of nucleic acid hybridization (Sutton, 1971) (b) analysis of heteroduplex DNA (Crosa et al., 1973) (c) isolation of rapidly annealing regions from single stranded DNA (Shishido and Ando, 1972; Sutton and Walker, 1972) (d) analysis of superhelical DNA (Mechali et al., 1973; Beard et al., 1973; Germond et al., 1974; Weigand et al., 1975; Godson, 1973).

Although the usefulness of this enzyme as a selective reagent has been recognized in its wide spread use but yet incompletely explored is the use of such enzymes to detect and

characterize small variations in the secondary structure of nucleic acids. The kinds of variation in secondary structure, particularly those of biological significance are e.g. the variation that would seem to be inherent in DNA that is being transcribed, replicated and recombined. An early initiation event in genetic recombination is the uptake by a recipient homologous double stranded molecule of a redundant single strand displaced from a homologous donor (Meselson and Radding, 1975). In an attempt to test the hypothesis that superhelicity itself might promote the uptake of homologous single strands Holloman et al. (1975) have investigated the association of single stranded fragments with the superhelical form of bacteriophage ϕx 174 DNA. It has been concluded that superhelical DNA spontaneously takes up homologous single stranded fragments forming a structure which is akin to D loops seen in replicating mitochondrial DNA (Kasumatso and Vinograd, 1974) which presumably is an intermediate in genetic recombination. The action of S_1 nuclease on this structure has been described and it has been shown that S_1 nuclease can stabilize the association of the donated strand with the relaxed recipient DNA. Shenk et al. (1975) have reported a biochemical method using S_1 nuclease to accurately map the location of mutational alterations in simian virus 40 (SV40) DNA. S_1 nuclease can be used to map deletions of between 32 and 190 base pairs. To map a deletion a mixture of unit length linear DNA, prepared from SV40 deletion mutant

and its wild type parent are denatured and reannealed to form heteroduplexes. S_1 nuclease can cut such fragments whose lengths correspond to the position of the deletion. Similarly specific fragments are produced when S_1 nuclease cleaves a heteroduplex formed from the DNA of SV40 temperature sensitive mutants and either their revertants or wild type parents. If, as is likely, the base sequence of the temperature sensitive mutants DNA differs from the wild by a single base change, this method could be useful for accurately mapping point mutations, thereby making reliance on genetic recombination unnecessary. S_1 nuclease mapping procedure is particularly important in that it permits the detection and location of deletions and insertions that are too small to be visualized by conventional electron microscopic analysis of heteroduplex DNAs. Dodgson and Wells (1977), using a series of synthetic DNA molecules containing heteroduplexes of increasing length, showed that S_1 nuclease would cleave at a region containing no fewer than three adjacent mismatched bases. Most pertinently, single base mismatches were cleaved very inefficiently. The possibility of S_1 nuclease nicking duplex ϕx 174 DNA molecules containing single base mismatches has been explored by Silber and Loeb (1981). Three biochemical and biological assays were established to identify any single base mismatch specific endonucleolytic activity of S_1 nuclease. The experiments were designed to demonstrate either preferential degradation or the

appearance of new physical or biological forms of ØX174 heteroduplex DNA. These involved the use of templates containing multiple single base mismatches or defined nucleotide mispairings. It was concluded that both S_1 nuclease as well as the single strand specific endonuclease from Neurospora crassa did not act preferentially at the site of the single base mismatch.

BND CELLULOSE CHROMATOGRAPHY

Current concepts of genetic processes such as DNA replication, recombination and repair involve the generation of DNA molecules having single stranded regions. Therefore, techniques that can discriminate such regions and can be used to fractionate such molecules will be of immense use. Chromatography of DNA on benzoylated naphthoylated DEAE (BND) cellulose was originally developed with these ideas in view (Gillam et al., 1967; Iyer and Rupp, 1971). Although all parameters influencing the affinity of nucleic acids to BND cellulose are not known, studies involving phage and bacterial DNA and RNA and replicating human cellular DNA have suggested a preferential interaction of BND cellulose with single stranded regions of nucleic acids. Double stranded DNA elutes from BND cellulose with 1M NaCl in 0.01M Tris HCl pH 7.5 and 10^{-4}M EDTA. DNA with single stranded regions requires 50% formamide or 2% caffeine with 1M NaCl. It has been noted that the caffeine concentration at which DNA is eluted from BND cellulose is a function of the degree of single strandedness (Iyer and Rupp, 1971). Adherence to BND cellulose is now

considered to be the result of at least two different properties: (a) Binding of single stranded regions or single strand breaks in DNA and (b) Binding of aromatic residues directly to the resin (Karran et al., 1977).

Strauss and colleagues (1975) have been able to separate DNA growing points in cultured human cells from the bulk of the DNA by chromatography on BND cellulose since such growing points have single stranded regions which adhere to the resin. The newly synthesized DNA produced by cells inhibited with hydroxyurea is rich in such single stranded regions because of the inability of newly made DNA to elongate in the presence of inhibitor. Non replicating bulk DNA will wash through the column with NaCl. In the presence of inhibitor the incorporation of labelled precursors into non replicating DNA will be minimal in the absence of damage. DNA repair can therefore be measured directly from the NaCl eluate of a BND cellulose column. DNA containing single stranded regions adhere to the column and are eluted with formamide and can be used to measure repair in the growing point region. Using this procedure these authors could not detect DNA repair in N-acetoxy-2-acetylamino-flourene treated lymphoblasts derived from an individual with the disease xeroderma pigmentosum, although methylmethane sulfonate induced repair was observed in the cells. Hogan et al.(1981) have used BND cellulose to study the binding of benzo-(a)-pyrene metabolite, the diol

epoxide r-7, t-8-dihydroxy-t-9, 10 oxy-7, 8, 9, 10 tetrahydro benzo-(a)-pyrene (BPDE) to denatured and native DNA. An equimolar mixture of both types of DNAs was reacted with BPDE and separated on BND cellulose. It was found that denatured DNA had 3.6 times more affinity for BPDE than native DNA.

INTERACTION OF RIBOFLAVIN WITH DNA

Visible light in the presence of riboflavin and oxygen is lethal to animal and human cells in tissue culture and induces mutations in micro-organisms and mammalian cells (Bradley and Sharkey, 1977; Webb et al., 1967). These effects indicate an alteration in nucleic acid structure. Changes in physico-chemical properties of DNA illuminated in the presence of riboflavin have been reported. These include a decreased sedimentation coefficient, a decrease in melting temperature and an increase in buoyant density (Speck et al., 1975; Speck et al., 1976). These observations are of interest in view of the fact that phototherapy for the treatment of neonatal hyperbilirubinaemia to reduce bilirubin levels is a widely used therapeutic manoeuvre (Behrman et al., 1974). The exact basis of the photo induced reaction is not known. One of the observed concomitants of phototherapy is a great reduction in the riboflavin level of treated infants (Sisson). This can be explained on the basis that the wavelength (450 nm) for maximum photodecomposition of

bilirubin corresponds to one of the absorbance peaks of riboflavin (Speck et al., 1975). On the other hand it is well known that lumiflavin and lumichrome are the photodegradation products of riboflavin. A chemically very active semiquinone type free radical is formed by the reduction of lumiflavin with visible light or other reagents (Kuratomi and Kobayashi, 1977).

The juxtaposition of riboflavin and its derivatives with nucleic acids in living cells is questionable. However it is not impossible that a biochemical as ubiquitous as riboflavin may exist in the nuclear material, possibly as coenzyme such as flavin adenine dinucleotide or flavin mononucleotide, which are also photosensitizers (Korycka-Dahl and Richardson, 1978). The physicochemical changes in DNA on interaction with riboflavin have been investigated further. It has been shown that the vitamin causes the degradation of guanine residues possibly by the singlet oxygen as riboflavin is a good singlet oxygen generator (Speck et al., 1976). If a mechanism involving interaction between riboflavin or one of its degradation products and guanine moieties of DNA is to be considered then intercalation of flavin between guanine and another base of DNA may have to occur (Kuratomi and Kobayashi,1977). Some support for the intercalation model comes from studies on the formation of superoxide anion during riboflavin sensitized photooxidation of nucleic acids. It was found that at the same molar concentration of nucleotides the rate of photogeneration of superoxide anion was about eight

times faster in the presence of DNA then in the presence of RNA (Korycka-Dahl and Richardson, 1980). It may be, that the more ordered structure of DNA favours an interaction, such as intercalation between the DNA and riboflavin. Further, whether the increase in buoyant density of DNA illuminated in the presence of riboflavin results from a structural alteration of DNA which occurs when the guanine residue is destroyed with simultaneous scission of the phosphodiester backbone has still not been conclusively determined.

In the present study we have used S_1 nuclease hydrolysis and BND cellulose chromatography with a view to investigate further the following points: (i) whether riboflavin intercalates into DNA as an initial step in the interaction (ii) whether single stranded regions or breaks are generated in DNA on photoillumination with riboflavin.

INTERACTION OF QUERCETIN WITH DNA

It is now generally accepted that environmental factors play a major part in human carcinogenesis. Although much attention has been focussed on industrial pollutants, these alone cannot be entirely responsible for human cancers. Of late, there has been increasing interest in naturally occurring compounds, ingested as part of the normal diet, which could be potentially mutagenic/carcinogenic (Wynder and Gori, 1977). One such class of compounds is the flavonoids which occur, sometimes in very large amounts,

in a wide range of food plants including many fruits, vegetables and tea. Onion (Allium cepa) contains about 11-24 gm of quercetin per kg fresh weight of outer epidermis (Hermann, 1976). There is also considerable interest in the flavonoid derivatives as possible non nutritive sweeteners. Quercetin and other flavonoids occur free or in conjugated form in the outer tissues, skin, or peel of fruits, tubers and roots. Since mutation tests using microbes have demonstrated that most typical carcinogens are mutagenic, microbial mutation tests are often used as a convenient screening method for environmental carcinogens (McCann et al., 1975; Poirier and Weisberger, 1979). There are many flavonoids in plants, and the mutagenicities of more than seventy naturally occurring flavonoids have been tested (Nagao et al., 1978). Of these, quercetin was the strongest mutagen, followed by kaempferol, rhamnetin, galangin, isorhamnetin and fisptin (Sugimura et al., 1977; Bjeldanes and Chang, 1977; Brown, 1980; Brown and Doetrich, 1979; Mardigree and Epler, 1978; Macgregor and Jurd, 1978; Nagao et al., 1981). These mutagenic flavonoids have hydroxy groups at the 3 and 5 positions. All these compounds except quercetin required metabolic activation by rat liver enzymes and the mutagenicity of quercetin was further enhanced by rat liver enzymes. S. typhimurium TA98 was more sensitive than TA 100 (Ames test) to all these flavonoids except woganin, which has no hydroxy group at the 3 position and which showed strong mutagenicity to TA 100 with metabolic activation (Nagao et al., 1978).

Besides the microbial systems, flavonoids, especially quercetin and kaempferol have also been tested for mutagenicity in higher systems such as rats, hamsters and drosophila. Quercetin and quercetrin were not found carcinogenic when fed to rats as 1% of the diet (Ambrose et al., 1952). Also, tests in ACI rats showed no carcinogenicity of quercetin (Hirono et al., 1981). However, previously Pamukco et al. (1980) reported evidence for the carcinogenicity of quercetin in Norwegian strain rats which were fed on a basal grain diet containing 0.1% quercetin. Quercetin and its glycoside rutin were tested for carcinogenicity in nnn inbred golden hamsters by Morino et al. (1982). Quercetin was not carcinogenic when given at the concentration of 4% and 1%. Even with the administration of 1% croton oil after 1% quercetin there was no increase in tumor incidence. Watson (1982) has studied the mutagenic activity of quercetin and kaempferol in Drosophila melanogaster. Frequencies of recessive sex linked lethals induced by quercetin and kaempferol in Drosophila sperm was determined. Significant increases were found in the frequencies of recessive lethals. These findings were considered to reinforce the positive results found in mammalian test systems since Drosophila appears to possess similar metabolizing capacities to those found in mammals. It has been estimated that the average daily intake of flavonoids in the American diet is about one gm and thus there is clearly a potential hazard.

MATERIALS AND METHODS

MATERIALS

Animals used for the study

Colony bred adult male albino rabbits weighing 1.2-1.8 Kg purchased locally were used for studies with berenil.

Chemicals

The following chemicals were obtained commercially and used without further purification. The chemicals were obtained from the sources given against their names.

Chemical	Source
Acridine orange	Searle Scientific Services, Bucks, England.
Acriflavine	Chemitron Laboratories, India.
Acrylamide	Sisco Research Laboratories, India.
Actinomycin D	Sigma Chemical Co.
Adenosine triphosphate	Sigma Chemical Co.
Agarose	Sigma Chemical Co.
L-Alanine	Sigma Chemical Co.
Ammonium per sulfate	May and Baker Ltd., England.
Benzoylated naphthoylated DEAE cellulose (BND-cellulose)	Sigma Chemical Co.
Berenil	Dr. D.P. Burma, B.H.U., Varanasi, India.
Bovine serum albumin	Sigma Chemical Co.
Bromophenol blue	E. Merck, Germany.

Casamino acids	BDH, Poole, England.
Caffeine	Sigma Chemical Co.
Chloroquine phosphate	I.D.P.L., India.
Creatine	Sigma Chemical Co.
Deoxyribonucleic acid (Calf thymus)	Sigma Chemical Co.
Deoxyribonucleic acid (from bacteriophage lambda)	Sigma Chemical Co.
Deoxyribonucleic acid (from plasmid pBR322)	Sigma Chemical Co.
DNase I	Sigma Chemical Co.
2-Deoxyribose	Sigma Chemical Co.
Dimethyl sulfate	May and Baker Ltd., England.
Dimethyl sulfoxide	B.D.H., England.
Diphenylamine (recrystallized from boiling hexane before use)	B.D.H., England.
Ethidium bromide	Sisco Research Laboratories, Bombay, India.
Ethylene diamine tetra acetic acid (disodium salt, EDTA)	B.D.H., England.
Formamide	E. Merck, Germany.
Fructose (AR)	Glaxo Laboratories (India).
Glucose (AR)	B.D.H., India.
Glycerol	B.D.H., India.
Heparin	Sigma Chemical Co.
Hydroxyapatite (HA)	Sisco Research Laboratories (India).
N-N' methylene bisacrylamide	B.D.H., Poole, England.

Nuclease S_1 (from *Aspergillus oryzae*)	Boehringer Mannheim, (Germany).
Perchloric acid	B.D.H., India.
Quercetin	Dr. Asifuzzaman Siddiqi, AMU, Aligarh.
Riboflavin	E. Merck, Germany.
Sepharose 6B	Pharmacia Fine Chemicals (Sweden).
Spermine	Dr. T.A. Bickle, University of Basel, Switzerland.
Streptomycin sulfate	Glaxo Laboratories, India.
N-N-N' -N' tetramethyl ethyl diamine (TEMED)	Fluka, Switzerland.
2,4,6 Trinitrobenzene sulfonic acid (TNBS)	Sigma Chemical Co.
Tris hydroxymethylamino methane (Tris)	B.D.H., England.
Trypsin	Sigma Chemical Co.
Tyrosine	Sigma Chemical Co.

METHODS

Hydroxyapatite chromatography

Hydroxyapatite was suspended in phosphate buffer (0.01M pH 7.0). The fine particles were removed and the slurry, mainly containing coarse particles, was poured into a column, of 1 cm cross section. The stop-cock was opened and sufficient amount of fluid was allowed to pass to obtain a 3 cm bed. DNA samples (2.5 ml containing 1 mg DNA) were applied and the elution started with a stepwise gradient of phosphate buffer (pH 7.0). Generally 3 ml fractions were collected at the rate of 10 ml/hour.

The DNA eluted in various fractions was determined by the diphenylamine reaction (Schneider, 1957) or spectrophotometrically by reading the absorbance at 260 nm. To a 1 ml aliquot 2 ml of diphenylamine reagent (freshly prepared by dissolving 1 gm of crystalline diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in boiling water for 20 minutes and the intensity of blue color was read at 600 nm.

Hydroxyapatite chromatography of DNA with the various intercalating and non intercalating agents was carried out as follows: 1 ml DNA in TNE was mixed with 1 ml of TNE and 0.5 ml of a 10^{-3}M solution of EtBr (396 ug/ml) to give a DNA base pair dye molar ratio of 3:1. The mixture was left at room tempera-

ture for 1 hour before applying to the hydroxyapatite column. Elution with increasing molarity of phosphate buffer was performed as described above. Chromatography with other drugs and dyes was done in a similar way.

The amount of DNA in various fractions was determined as already described. The various dyes in each fraction were determined by reading the absorbance at their corresponding max. values; ethidium bromide - 480 nm; acriflavin - 440 nm; actinomycin D - 440 nm and acridine orange - 440 nm. Spermine was determined by the method of Synder and Sobocinski (1975) using 2,4,6 trinitrobenzene sulfonic acid (TNBS) and berenil was determined by its absorption at 370 nm or by its color reaction with diphenylamine which has been described in this dissertation. The dye eluting in the fractions containing DNA was taken as that bound to DNA and was quantitated using its extinction coefficient.

The molar extinction coefficient for ethidium bromide bound to DNA was determined as follows: The absorbance at 480 nm was measured for solutions of DNA at a concentration of 2 mg/ml in TNE to which ethidium bromide had been added over a concentration range of 5-40 mg/ml. A linear plot was obtained and the molar extinction coefficient was determined to be $4 \times 10^3 M^{-1} cm^{-1}$. Ethidium bromide alone gave a molar extinction coefficient of $7 \times 10^3 M^{-1} cm^{-1}$.

Scatchard analysis of the degree of ethidium bromide binding to DNA

The degree of ethidium bromide binding to DNA was determined spectrophotometrically. In a total volume of 8 ml TNE 1 umole of ethidium bromide (1 ml) was mixed with various concentrations of DNA (between 0.3-3 umole bp). The optical density of the solution was read at 480 nm. The number of moles of ethidium bromide bound/mole of DNA bp (r) and the concentration of the unbound dye (c) was calculated by the procedure described by Peacocke and Skerett (1956).

Estimation of berenil by its color reaction with diphenylamine

To a 1 ml solution of berenil in water was added 2 ml of diphenylamine reagent prepared freshly as already described. After a few minutes a pink colour develops which remains stable for at least 24 hours at room temperature. The absorbance of the various tubes was read at 530 nm as the colored product showed its absorption maximum at this wavelength. When the effect of various additions on the method was tested, the substances were dissolved in water and mixed with berenil solution before addition of the diphenylamine reagent.

Preparation of purified erythrocytes and erythrocyte ghosts

A preparation containing purified RBCs was obtained from rabbit blood as follows: 1 ml of heparinised blood was centrifuged at 3000 rpm for 20 minutes to separate plasma in the form

of supernatant and the particulate material containing RBCs in the precipitate. The supernatant was discarded and the precipitate was washed repeatedly with normal saline (0.9% NaCl) prior to centrifugation till a clear supernatant is achieved and purified RBCs are left in the precipitate.

Erythrocyte ghosts were obtained from the purified RBCs preparation as follows: The purified RBCs obtained from 1 ml of blood were suspended in 5 ml of 5mM Tris HCl pH 7.5 and centrifuged at 12000 rpm for 30 minutes. The pellet obtained was extensively washed repeatedly with the same buffer prior to centrifugation till hemoglobin depletion from erythrocytes is complete. Erythrocyte ghosts or RBC membranes are obtained in the form of a yellowish white pellet.

Assay of S_1 nuclease

S_1 nuclease was assayed by estimating the acid soluble nucleotides released from DNA as a result of enzymatic digestion. The reaction mixture in a final volume of 1 ml contained 500 ug of substrate (native, denatured or modified DNAs), 0.1M acetate buffer pH 4.5, 1 mM $ZnSO_4$, water and enzyme. The reaction mixture was incubated at 40°C (unless otherwise specified) for the desired period of time. At the end of the incubation period the reaction was terminated by the addition of 0.2 ml of 10 mg/ml bovine serum albumin, mixed thoroughly by shaking and 1 ml of ice cold 14% perchloric acid. The tubes were immediately

with approximately 150 ml of 0.3M NET. A jacketed column was used and maintained at 10°C with the help of circulating ice cold water. 8 ml of native calf thymus DNA (16 mg in TNE) was loaded on the column. DNA was step eluted with 72 ml of 0.3M NET, 36 ml of 1M NET + 50% formamide or 2% caffeine. 24 fractions of 3 ml each of 0.3M NET and 18 fractions of 2 ml each of 1M NET and 1M NET + 2% caffeine (or 1M NET + 50% formamide) were collected at the rate of 10-12 ml/hour. DNA in 0.2 ml of each fraction was estimated by the diphenylamine reaction. Peak fractions of 1M NET were pooled and dialyzed overnight against TNE (0.1M NaCl or 0.01M NaCl). The completely double stranded DNA so obtained was stored frozen in batches.

(b) Chromatography of alkylated DNA on BND cellulose

DNA without single stranded breaks or regions was obtained by prechromatography of calf thymus DNA on BND cellulose as described above. 100 ug DNA samples were alkylated for 1 hour with sufficient dimethyl sulfate (diluted 10 fold in TNE) to obtain the desired DNA nucleotide/DMS motar ratio. The alkylated DNA was directly applied to the BND cellulose column (1x2.5 cm) pre-equilibrated with 0.3M NET. The elution was carried out with 0.3M NET, 1M NET and 1M NET + 2 % caffeine (or 50 % formamide). 2 ml fractions were collected at a rate of 8 ml/hour. DNA was measured by the diphenylamine method of Burton (1956).

DNA gel electrophoresis

For the analysis of DNA samples, a vertical slab gel electrophoresis system was employed. To the reaction mixture was added 5 ug pBR322 DNA; 0.34 ug riboflavin (DNA/riboflavin = 18:1) and TNE (q.v.) and incubated as desired. The sample buffer (containing 50% glycerol (v/v), 0.2% sodium dodecylsulfate and 0.5% bromophenol blue in 5 x TBE buffer) was added and applied to 0.5 cm slots of the 1.2% agarose gel slabs (0.15 x 16 x 16 cm). The volume of the sample buffer was 1/5th of the total reaction mixture. The electrophoresis buffer used was 0.45M Tris borate, pH 8.3, 20 mM EDTA (TBE buffer). It was diluted 5 times prior to use. The electrophoresis was performed at room temperature at 4mAmp/slot till the dye migrated through the whole length of the gel. The gels were stained with 1 ug/ml ethidium bromide. Flouresecent bands of DNA in gels were visualized by illumination from top with a short wave ultravoilet lamp at 254 nm and were photographed using Orwo black and white film (ASA-400).

Sepharose column chromatography

Analytical column of sepharose 6B was prepared according to the standard procedure supplied by the Pharmacia Fine Chemicals. A required amount of Sepharose 6B to give a bed volume of 2.2 x 22 cm was allowed to swell in a suitable amount of double distilled water for 6 hours in boiling water bath. A

glass column previously cleaned with chromic acid, detergent and distilled water was mounted vertically. One third of the column length was filled with the operating TE buffer (0.01M Tris HCl pH 7.5, 10^{-3}M EDTA and 0.2M NaCl) and then slurry of the gel (deaerated under vacuum) was poured into it with the help of a glass rod. The column was left standing for an hour till the gel had settled. After this period, the stopcock was opened with slowly increasing rate. After a constant rate of flow higher than required for final elution was achieved, the rate was adjusted to that required. The column was washed with a volume of TE buffer (3-4 times the bed volume) to ensure no further shrinking of the bed height during the experiment. To determine the uniform packing and to determine the void volume (V_0) of the column 0.2 percent solution of Blue dextran (1 ml) was passed through the column. The volume of the dye was 2 percent (or lower) of the total bed volume of the column.

Estimation of DNA and acid soluble nucleotides

DNA nucleotides made acid soluble were determined by the diphenylamine method of Schneider (1957) or spectrophotometrically by reading the absorbance at 260 nm. To a 1ml aliquot, 2 ml of diphenylamine reagent (freshly prepared by dissolving 1 gm of recrystallized diphenylamine in 100 ml of glacial acetic acid (AR grade) and 2.75 ml of conc. H_2SO_4 (AR grade)) was added. The tubes were heated in a boiling water

bath for 20 minutes. The intensity of blue color was read in Spectronic 20 Bausch and Lomb spectrophotometer. To determine the acid soluble material spectrophotometrically, an aliquot of the supernatant was suitably diluted and read at 260 nm in Spectronic 21 UVD Bausch and Lomb spectrophotometer against a suitable blank. In some experiments DNA was also determined by the diphenylamine modified procedure of Burton (1956). This procedure considerably increases the sensitivity of the reaction. To 1 ml aliquot was added 1 ml of 1N perchloric acid and the mixture heated at 70°C for 15 minutes. To this were added 0.1 ml of 5.43 mM acetaldehyde and 2 ml of Burton's diphenylamine reagent (freshly prepared by dissolving 1.5 gm of recrystallized diphenylamine in 100 ml glacial acetic acid (AR grade) and 1.5 ml of conc. H_2SO_4 (AR grade)). The developed blue color was read at 600 nm.

RESULTS AND DISCUSSION

HYDROXYAPATTTE CHROMATOGRAPHY OF NATIVE DNA WITH INTERCALATING AND NON INTERCALATING AGENTS

Figure 1(a) shows the chromatographic pattern of standard native and denatured DNA samples on hydroxyapatite. Native DNA is eluted in two fractions with 0.25M and 0.3M phosphate buffer. Denatured DNA showed poor recovery as has already been noted (Bernardi, 1971). Figures b,c,d,e are similar experiments where native DNA was first incubated with the intercalating dyes ethidium bromide, acriflavine, actinomycin D and acridine orange respectively before loading on the column. It is seen that in all cases some of the dye is eluted along with the DNA in the same fractions. In addition, the DNA is eluted at a lower phosphate molarity, i.e., with 0.2M and 0.25M phosphate buffer, suggesting a lower affinity of DNA to hydroxyapatite in the presence of intercalating dyes.

Figure 2 shows HA chromatography of native DNA with compounds, viz., berenil and spermine known to bind to DNA by non intercalative means (Waring, 1970), presumably through electrostatic interactions. Both the ligands are completely eluted at a much lower phosphate molarity than DNA. The fractions containing DNA do not contain any ligand. These results indicate that the dyes which bind by intercalation elute from HA columns complexed to DNA. On the other hand drugs which bind to DNA by electrostatic interaction elute earlier than the macromolecule. The lower affinity of DNA-intercalating agent

Fig. 1 Hydroxyapatite chromatography of native DNA with intercalating agents

In a total volume of 2.5 ml TNE 1 mg DNA was mixed with the appropriate concentration of intercalating agents used to give a DNA bp:dye molar ratio of 3:1. The solution was left at room temperature for 1 hour before applying to the hydroxyapatite column. Stepwise elution was performed with phosphate buffer pH 7.0.

See text for further details.

(a) x - - - x denatured DNA; . _____ . native DNA

(b) native DNA with EtBr

(c) native DNA with acriflavine

(d) native DNA with actinomycin D

(e) native DNA with acridine orange

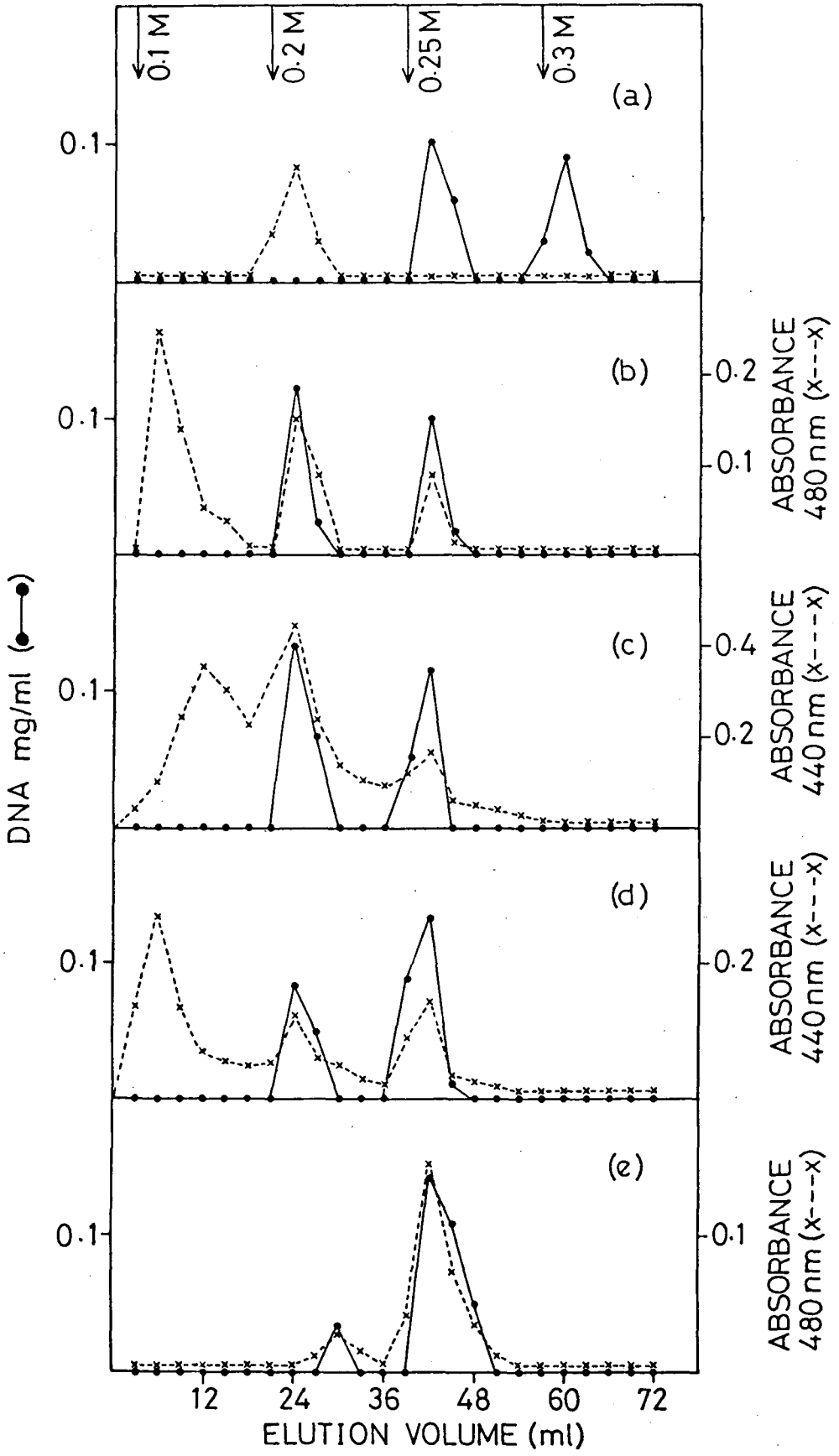
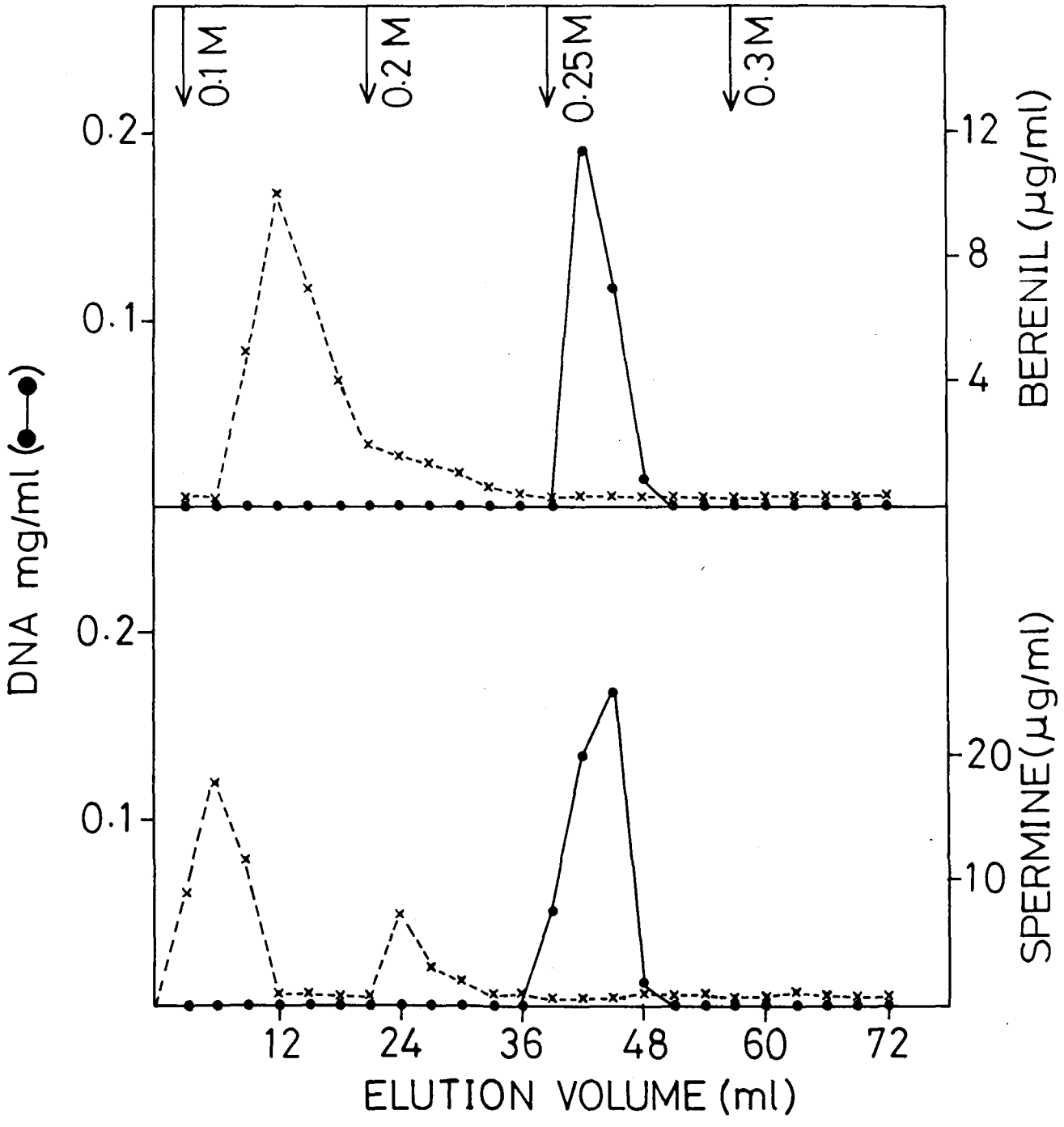

0.1 M
0.2 M
0.25 M
0.3 M
(a)
(b)
(c)
(d)
(e)
0.1
0.2
0.4
DNA mg/ml (●—●)
ABSORBANCE 480 nm (x---x)
ABSORBANCE 440 nm (x---x)
12
24
36
48
60
72
ELUTION VOLUME (ml)

Fig. 2 Hydroxyapatite Chromatography of native DNA with non intercalating agents.

Details for the experiment are the same as described for fig. 1. Methods for determination of spermine and berenil are given in the text.


0.1 M
0.2 M
0.25 M
0.3 M
0·2
0·1
12
8
4
BERENIL (µg/ml)
0·2
0·1
20
10
SPERMINE (µg/ml)
DNA mg/ml
12
24
36
48
60
72
ELUTION VOLUME (ml)

complex for HA observed in Fig. 1 is presumably due to the variation in the steric availability of nucleic acid backbone phosphates for interaction with hydroxyapatite (Martinson, 1973c). This will be discussed further.

The fact that part of DNA - intercalating agent complex elutes from HA columns at the same phosphate molarity as single stranded DNA suggested the possibility of DNA undergoing denaturation in the process. This was ruled out as follows: The fractions containing DNA in Fig. 1(b) were pooled and the ethidium bromide was removed from it by frequent extractions with 1:1 mixture of n-butanol and chloroform. The DNA was reloaded on an HA column and the elution performed in the usual manner. As shown in Fig. 3(a) all the DNA now elutes at 0.3M sodium phosphate buffer indicating its essential double stranded nature. Fig. 3(b) shows the effect of sodium chloride on the HA chromatography of DNA - ethidium bromide complex. The DNA-EtBr mixture was applied to the column in presence of 0.2M NaCl. All the DNA is eluted in one fraction only at 0.2M sodium phosphate; however no dissociation of ethidium bromide from it is seen indicating that the complex eluting from the column contains ethidium bromide mainly in the intercalated form with DNA.

Figure 4 shows the absorption spectrum of ethidium bromide and the peak fraction of DNA eluting at 0.25M sodium phosphate

Fig. 3(a) Rechromatography on hydroxyapatite of DNA containing fractions of fig. 1(b) after extraction of ethidium bromide

See text for details.

Fig. 3(b) Effect of sodium chloride on hydroxyapatite chromatography of DNA with ethidium bromide

DNA - EtBr complex was prepared as described in fig. 1 and in the text except that the solution contained 0.2M NaCl. The other details were the same as described for fig. 1.

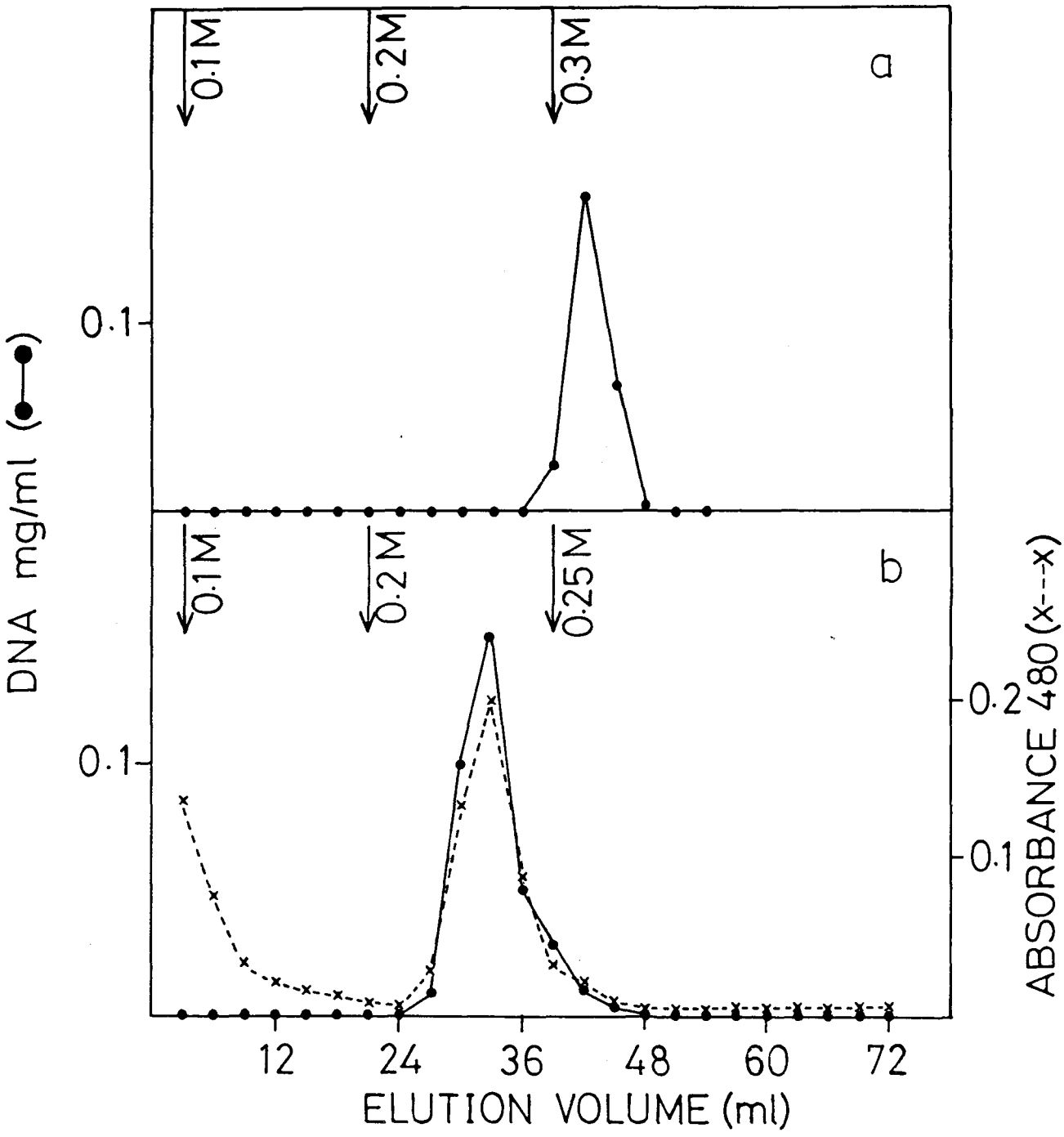
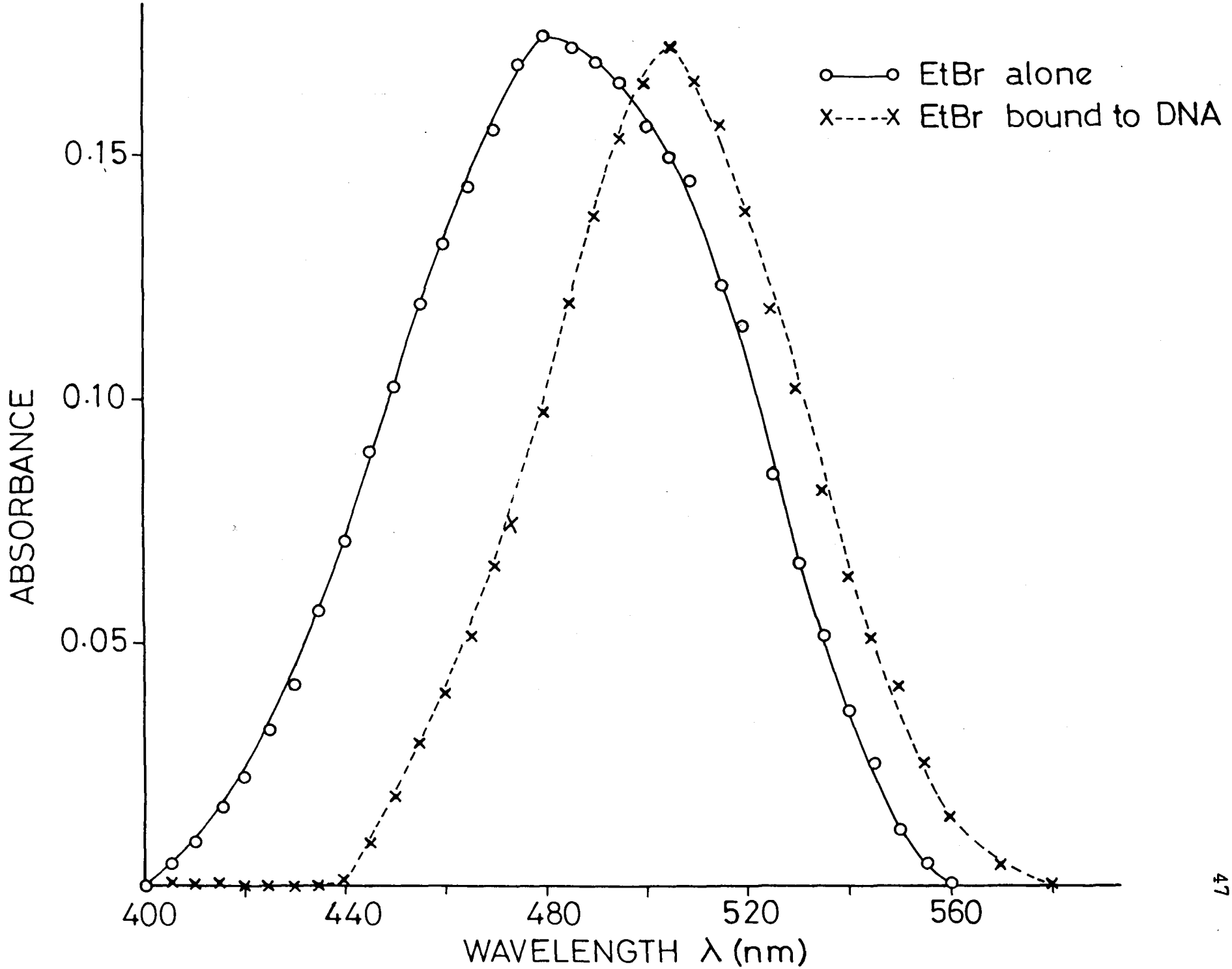

0.1M
0.2M
0.3M
a
0.1
0.1M
0.2M
0.25M
b
0.1
DNA mg/ml (●—●)
0.2
0.1
ABSORBANCE 480 (x--x)
12
24
36
48
60
72
ELUTION VOLUME (ml)

Fig. 4 Absorption spectrum of ethidium bromide alone and DNA - EtBr complex eluting with 0.25M sodium phosphate buffer in fig. 1(b)


EtBr alone
EtBr bound to DNA
0·15
0·10
0·05
ABSORBANCE
400
440
480
520
560
WAVELENGTH λ (nm)

in Fig. 1(b). A shift in the absorption maxima from 480 nm to 505 nm is observed which is identical to the shift reported by others upon binding of the drug to single and double stranded polynucleotides (Lepecq and Paoletti, 1967; Waring, 1965; Bittman, 1969) and is indicative of the formation of specific complexes of the drug with DNA. Thus, DNA is eluted from HA as a complex with ethidium bromide.

Scatchard analysis of ethidium bromide binding to DNA

The degree of binding of ethidium bromide to DNA was determined spectrophotometrically by the method of Peacocke and Skerett (1956) as described. A typical Scatchard analysis is shown in fig. 5 as a plot of r/c versus r. The letter r represents the number of moles of ethidium bromide bound/mole of DNA base pair and c is the concentration of the unbound dye. The general shape of the curve is indicative of complex binding. The stronger binding, characterized as intercalation, has been analyzed from the linear portion of the Scatchard plot at small values of r. A value of 0.33 moles of ethidium bromide/ mole of DNA base pair was determined.

In order to determine whether it is possible to quantitate and to determine the degree of binding by the HA chromatography procedure, the experiments shown in Table I were carried out. Increasing concentrations of ethidium bromide were allowed to interact with a fixed amount of DNA and subjected to

Fig. 5 Scatchard plot of the binding of ethidium bromide to calf thymus DNA

See text for details.

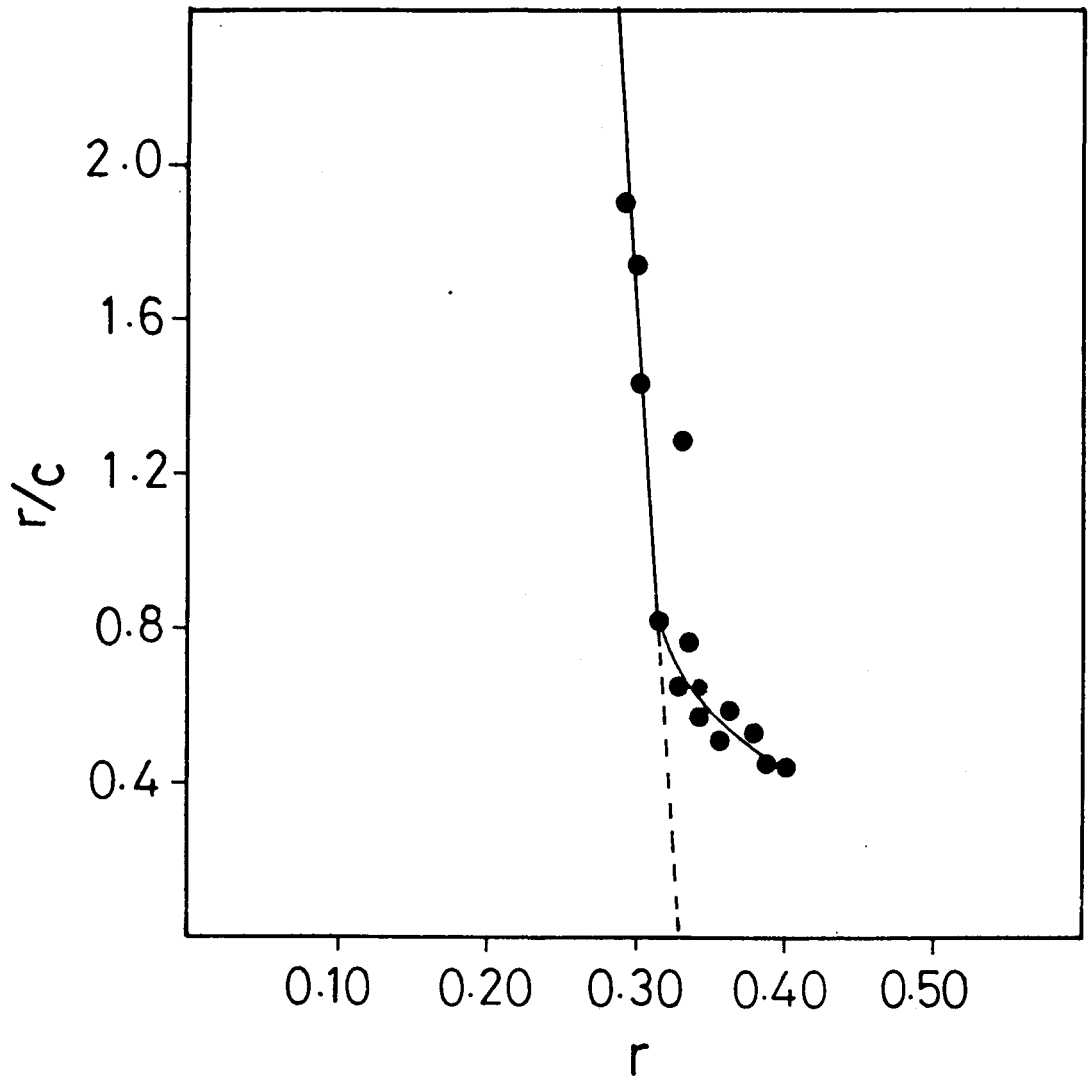

2·0
1·6
1·2
0·8
0·4
r/c
0·10
0·20
0·30
0·40
0·50
r

Table I

HYDROXYAPATITE CHROMATOGRAPHIC ANALYSIS OF ETHIDIUM BROMIDE BINDING TO DNA

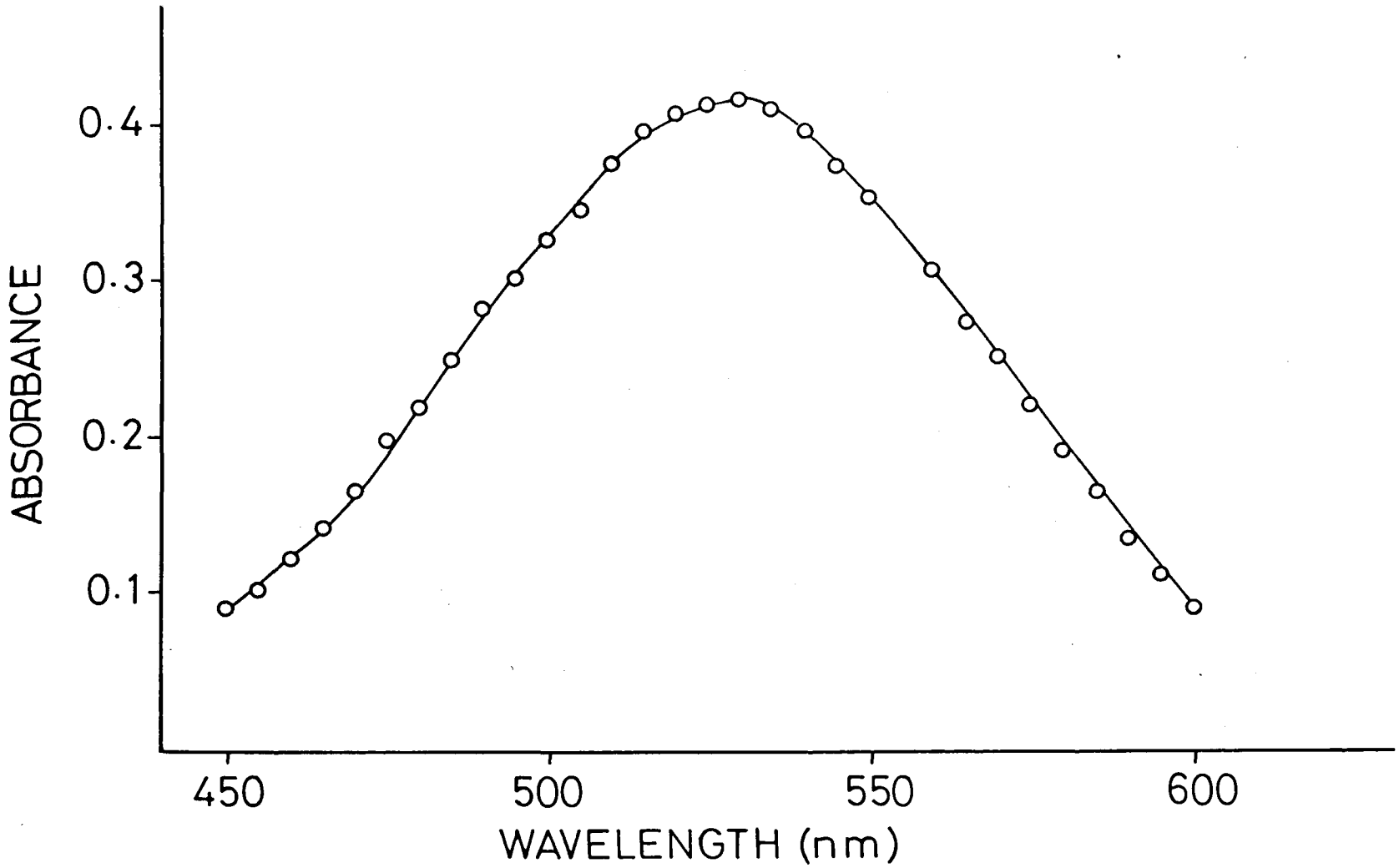
μmole EtBr	μmole DNA in base pair	DNA bp/EtBr molar ratio	Mole EtBr bound/ mole DNA bp
0.125	1.5	12:1	0.04
0.25	1.5	6:1	0.09
0.5	1.5	3:1	0.19
0.75	1.5	2:1	0.30
1.0	1.5	1.5:1	0.32

HA chromatography. The concentration of the dye bound to DNA was determined in the fractions containing DNA using a molar extinction coefficient of 4 x $10^3 M^{-1} cm^{-1}$ at 480 nm. As shown, 0.30 moles of ethidium bromide are bound/mole of DNA base pair at a DNA bp/dye molar ratio of 2:1. At a higher ethidium bromide concentration where a molar ratio of 1.5:1 was used, only a slight increase in the binding of the dye is observed. This value is in excellent agreement with the results obtained by Scatchard analysis. It is therefore, concluded that it is possible to determine the degree of binding of intercalating agents to DNA by the hydroxyapatite procedure.

Development of a color reaction for the determination of berenil

Berenil is a trypanocidal drug used for the treatment of trypanosomiasis in cattle in Africa. The generally used procedure for the estimation of berenil is the measurement of its absorption at 370 nm (Sinha et al., 1978; Newton, 1967). This method is not suitable for the estimation of the drug in biological fluids. We describe below a color reaction of berenil with diphenylamine which may eventually prove suitable for determining the drug concentrations in biological fluids. The details of the procedure have been described in the methods. Briefly, a solution in 1 ml of water of berenil is mixed with 2 ml of diphenylamine reagent. A pink color develops immediately

Fig. 6 Absorption spectrum of the color developed on reaction of berenil with diphenylamine.


ABSORBANCE
0·4
0·3
0·2
0·1
450
500
550
600
WAVELENGTH (nm)

which is stable for at least 24 hours. Fig. 6 is an absorption spectrum of the color which shows a maximum at 530 nm. Fig. 7 is the standard curve of berenil using the protocol described above and shows a linear response of the intensity of the color upto 40 ugs berenil. Fig. 8 shows a curve between berenil concentration and optical density at its absorption maximum of 370 nm. This shows a linear response upto a concentration of 20 ug/ml. Comparing figures 7 and 8 it is seen that whereas the absorption at max procedure is slightly more sensitive, the color reaction is applicable over a wider range of berenil concentration.

Table 2 shows the effect of diphenylamine concentration on the intensity of the color. Using a fixed concentration of berenil it was seen that the intensity of the color remained the same over a wide range of diphenylamine concentration. Effect of some substances present in biological fluids on the berenil-diphenylamine color reaction is shown in Table 3. No interference in the development of color is observed. Although more work needs to be carried out it appears that the method described above may prove to be a useful procedure for determining berenil in biological fluids and for pharmacokinetic studies.

Binding of berenil to various blood fractions

In order to test the applicability of berenil-diphenylamine

Fig. 7 Standard curve for berenil estimation by the diphenylamine procedure.

See text for details.

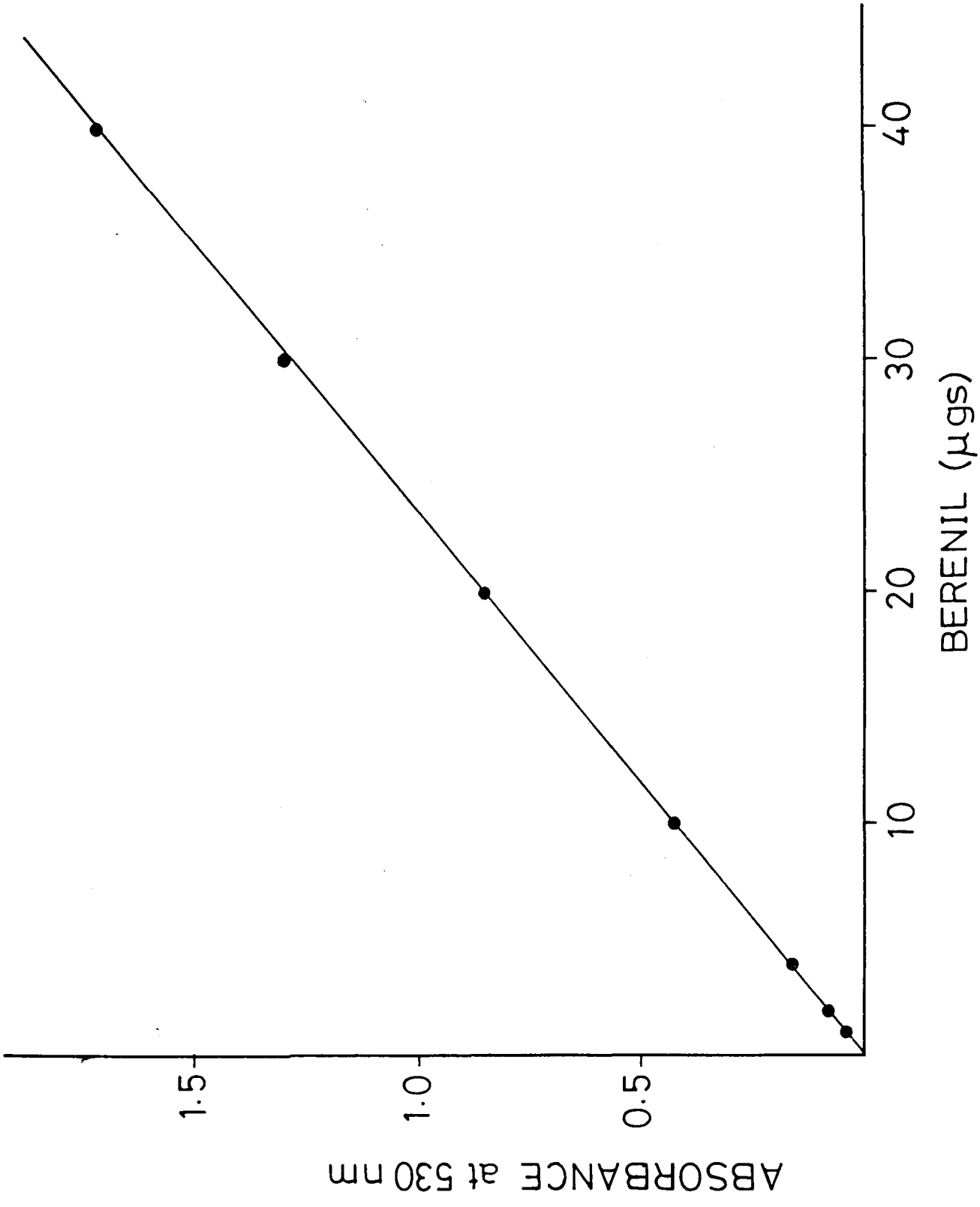

ABSORBANCE at 530 nm
0.5
1.0
1.5
10
20
30
40
BERENIL (µgs)

Fig. 8 Standard curve of berenil drawn by its absorption at 370 nm.

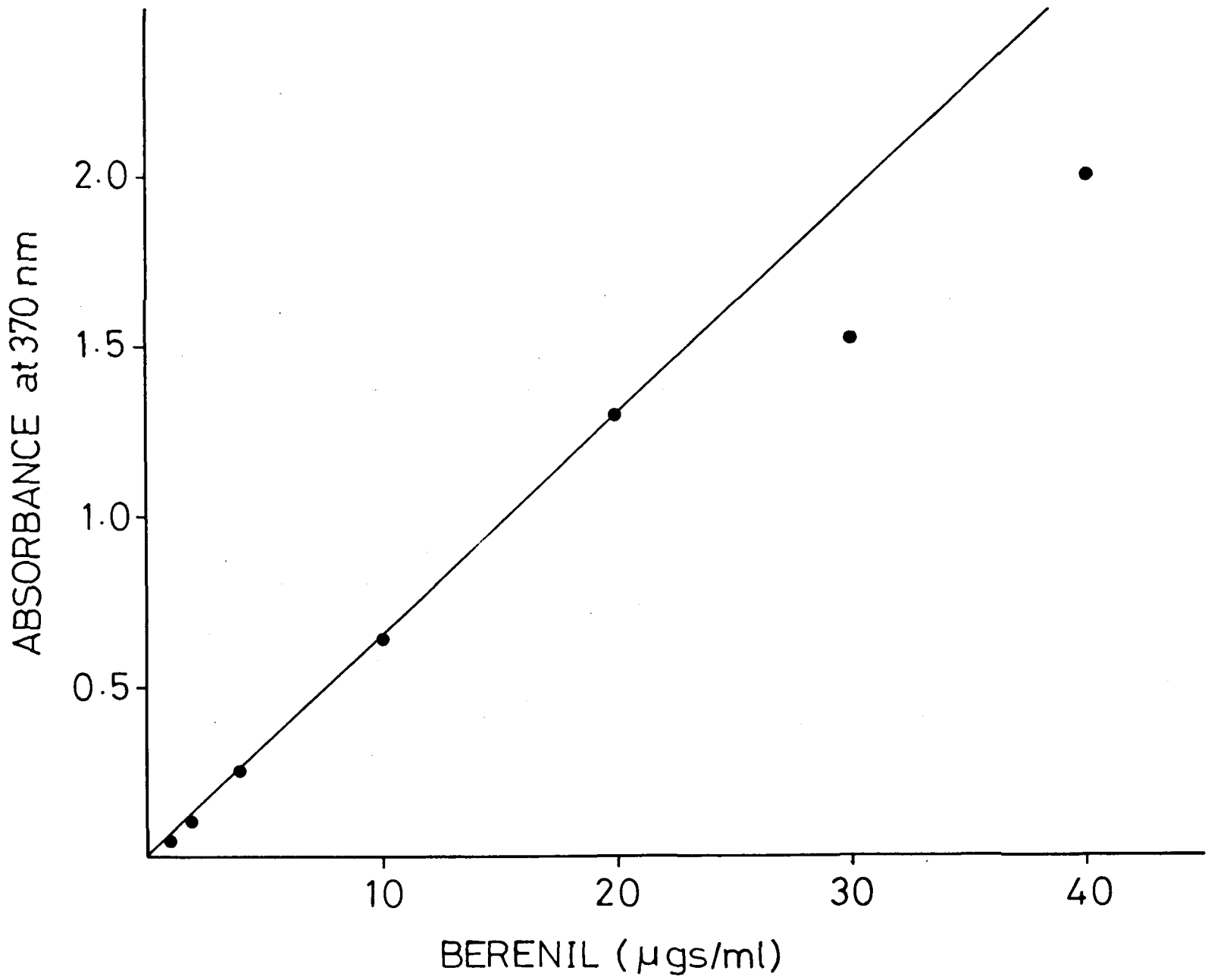

ABSORBANCE at 370 nm
2·0
1·5
1·0
0·5
10
20
30
40
BERENIL (µgs/ml)

Table II

EFFECT OF DIPHENYLAMINE CONCENTRATION ON THE INTENSITY OF THE COLOR DEVELOPED BY BERENIL-DIPHENYLAMINE COLOR REACTION

Concentration of Diphenylamine in the DPA reagent	Concentration of Berenil	O.D. at 530 nm
2.5 mg/ml	10 µg	0.44
5.0 mg/ml	10 µg	0.44
7.5 mg/ml	10 µg	0.43
10.0 mg/ml	10 µg	0.42
15.0 mg/ml	10 µg	0.42
20.0 mg/ml	10 µg	0.42
25.0 mg/ml	10 µg	0.48

color reaction with blood serum, a rabbit was injected intravenously with 20 mg of berenil/kg body weight. Blood samples were taken out at various time intervals ranging from 10 to 120 minutes. The drug was estimated by the color reaction after precipitating the serum with TCA. None of the samples showed the presence of berenil. At higher doses, similar results were obtained. These results suggested the possibility of the drug binding irreversibly to one or several of the blood fractions. Table III shows the results of an experiment where berenil was incubated in vitro, with various blood fractions and the recovery determined after precipitation of proteins with TCA. It is seen that whereas whole blood binds almost 100% berenil, binding to plasma and serum was only 30-40%. Purified RBCs bound 70% but RBC membranes did not show any binding. Haemoglobin, equivalent to 1 ml of blood bound 70% of added berenil. These results indicate that berenil binds only to haemoglobin and the various plasma proteins presumably through strong hydrophobic interactions.

Table III

EFFECT OF VARIOUS COMPOUNDS ON THE BERENIL-DIPHENYLAMINE COLOR REACTION

Compound tested	Concentration of the compound tested	Concentration of Berenil	D.D.at 530 nm
CONTROL	-	10 µg	0.56
ATP	10 µg	10 µg	0.56
GLUCOSE	10 µg	10 µg	0.56
FRUCTOSE	10 µg	10 µg	0.56
TYROSINE	10 µg	10 µg	0.56
CREATINE	10 µg	10 µg	0.56
BSA	10 µg	10 µg	0.56
L-ALANINE	10 µg	10 µg	0.56
CASAMINO ACIDS	10 µg	10 µg	0.56
SUCROSE	10 µg	10 µg	0.56
HEPARIN	10 µg	10 µg	0.56
2-DEOXYRIBOSE	10 µg	10 µg	0.56

Table IV

BINDING OF BERENIL TO VARIOUS BLOOD FRACTIONS

S.No.	Treatment	% recovery of Berenil
1	Berenil alone	100
2	Berenil + Heparinised blood	7
3	Berenil + Heparinised blood + Trypsin	12
4	Berenil + plasma	65
5	Berenil + serum	50
6	Berenil + Heparinised blood - centrifuge - supernatant	50
7	Berenil + Non-Heparinised blood - centrifuge - supernatant	10
8	Berenil + purified RBCs(from 1 ml of blood)	31
9	Berenil + RBC ghosts (from 1 ml of blood)	100
10	Berenil + Haemoglobin (150 mg)	38

DISCUSSION

It has been estimated that approximately 80% of human cancers occur due to chemical carcinogens. The exact mechanism by which chemicals cause cancer is not known but chemical carcinogens are known to react with DNA and a good correlation between mutagenesis and carcinogenesis has been established. It is therefore, important to determine the mode of interaction of various chemical mutagens. As has already been pointed out intercalation is the primary mode of interaction of many polycyclic aromatic mutagens with DNA (Fiel et al., 1979). Intercalation may also play an important role in protein - DNA interactions through the planar ring amino acids tryptophan, tyrosine and phenylalanine which present the possibility of intercalating between nucleic acid bases (Wells et al., 1980). Although many experimental techniques are available to determine whether a particular compound binds to DNA by intercalation, I have described a simple and rapid procedure for this purpose using hydroxyapatite chromatography.

The principal conclusions from the above experiments may be stated as follows: (i) when native DNA is chromatographed on HA complexed with drugs believed to bind to DNA by intercalation (ethidium bromide, acriflavine, actinomycin D and acridine orange) a lowered affinity of DNA to HA is observed resulting in its elution from HA columns at a relatively lower phosphate

molarity. Also, DNA is eluted from the columns as a drug - DNA complex, (ii) drugs that are believed to interact with DNA by mechanisms other than intercalation (spermine, berenil) do not show these effects, (iii) it may be possible to quantitate the binding of the intercalating drug to DNA by HA chromatography and to determine its degree of binding which agrees well with that determined by spectrophotometric data.

According to Martinson (1973c), for the variations in affinity of the various double stranded nucleic acids (ds-DNA, ds-RNA, RNA-DNA hybrid etc.) to hydroxyapatite, a number of possible mechanisms can be considered. Among these are included changes in adsorption affinity due to (1) variations in the interactions of the nucleic acid bases with hydroxyapatite, (2) variations in the charge densities of the different nucleic acids, (3) variations in the distribution of phosphates on the nucleic acids, (4) variations in the redistribution of ions and water molecules during adsorption of different nucleic acids, and (5) variations in the steric availability of the nucleic acid backbone phosphates for surface interactions. On the basis of various arguments he has further suggested that possibilities 1-4 can be considered to be of minor significance. On the other hand variations in the configurations of the sugar-phosphate helix exterior (no:5 above) appear to be principally involved in modifying the adsorption affinity of double stranded nucleic acids. Martinson (1973c)has also shown that

the phosphate groups of DNA protrude from the helix and are apparently relatively accessible for further interactions while the phosphate groups of both ds-RNA and DNA-RNA hydrid appear 'buried' in the surface of the helix. These observations correlate well with the finding that ds-RNA and DNA-RNA hydrid both have less affinity for hydroxyapatite than does ds-DNA and that ds-RNA and DNA-RNA hydrid chromatograph on hydroxyapatite very similarly to each other. It is now generally agreed that intercalation of planar ligands between DNA base pairs involves partial uncoiling of the double helix (Waring, 1970). Possibly intercalation causes a change in the configuration of the sugar-phosphate backbone of DNA resulting in an altered steric orientation or 'burial' of phosphate groups with reduced availability for surface interactions with hydroxyapatite.

Quantitation of ethidium bromide binding to DNA by hydroxyapatite

Upon addition of DNA to a solution of ethidium bromide a shift in the absorption spectrum of the dye from 480 - 520 nm is observed. With increasing amounts of DNA a well defined, reproducible isobestic point at 510 nm is obtained. These properties are indicative of the formation of specific complexes of the drug with DNA (Lepecq and Paoletti, 1967; Waring, 1965; Bittman, 1969). Use has been made of this metachromic shift to measure the drug/DNA

binding ratio by the spectrophotometric method of Peacocks and Skerett (1956). The general shape of the Scatchard plot (Scatchard, 1949) obtained is indicative of complex binding or more than one type of binding sites. The first and stronger binding is characterised as intercalation and is analyzed from the linear portion of the plot at small values of r (Fiel et al., 1979). The other types of binding is presumably related to electrostatic interaction between the dye and DNA since ethidium bromide carries a net positive charge. A value of 0.33 moles of ethidium bromide bound/mole of DNA base pair is obtained for intercalative binding.

Increasing amounts of ethidium bromide were complexed with DNA and chromatographed on HA. The amount of the dye bound to DNA was determined by using the extinction coefficient at 480 nm of ethidium bromide bound to DNA determined as described in 'Methods'. A value of 0.31 moles of the dye bound/mole of DNA base pair was obtained at the highest ethidium bromide concentration used. Since this value is close to that obtained by spectrophotometric procedure, it indicates that HA chromatography procedure may be used to quantitate the binding of intercalating agents to DNA.

Colorimetric method for the estimation of berenil

Berenil is a trypanocidal drug which contains strongly basic groups and is known to bind to DNA by nonintercalative means. It causes preferential inhibition of kinetoplast DNA synthesis and after a brief exposure of trypanosomes to berenil,

a marked decrease in the buoyant density of kinetoplast DNA is found with little effect on nuclear DNA (Lerman, 1964). Calf thymus DNA was found to bind one molecule of berenil per every 4-5 nucleotides and heat denaturation of DNA doubles the amount of drug bound. In neutral solutions the berenil molecule undergoes a rearrangement which results in the formation of 0-amino azo derivative. This breakdown product is without trypanocidal activity. This finding suggests that the spacing of amidino groups in berenil may be critical for the formation of complex with DNA.

The general method of estimating berenil is by measurement of its absorption in the near UV region at 370 nm. I have reported above a sensitive color reaction for this purpose. The chemical basis for the formation of pink color with diphenylamine is not known. However, some of the biological substances tested did not have any effect on the sensitivity of the color reaction. Therefore, the method has potential in following the berenil levels in blood serum etc.

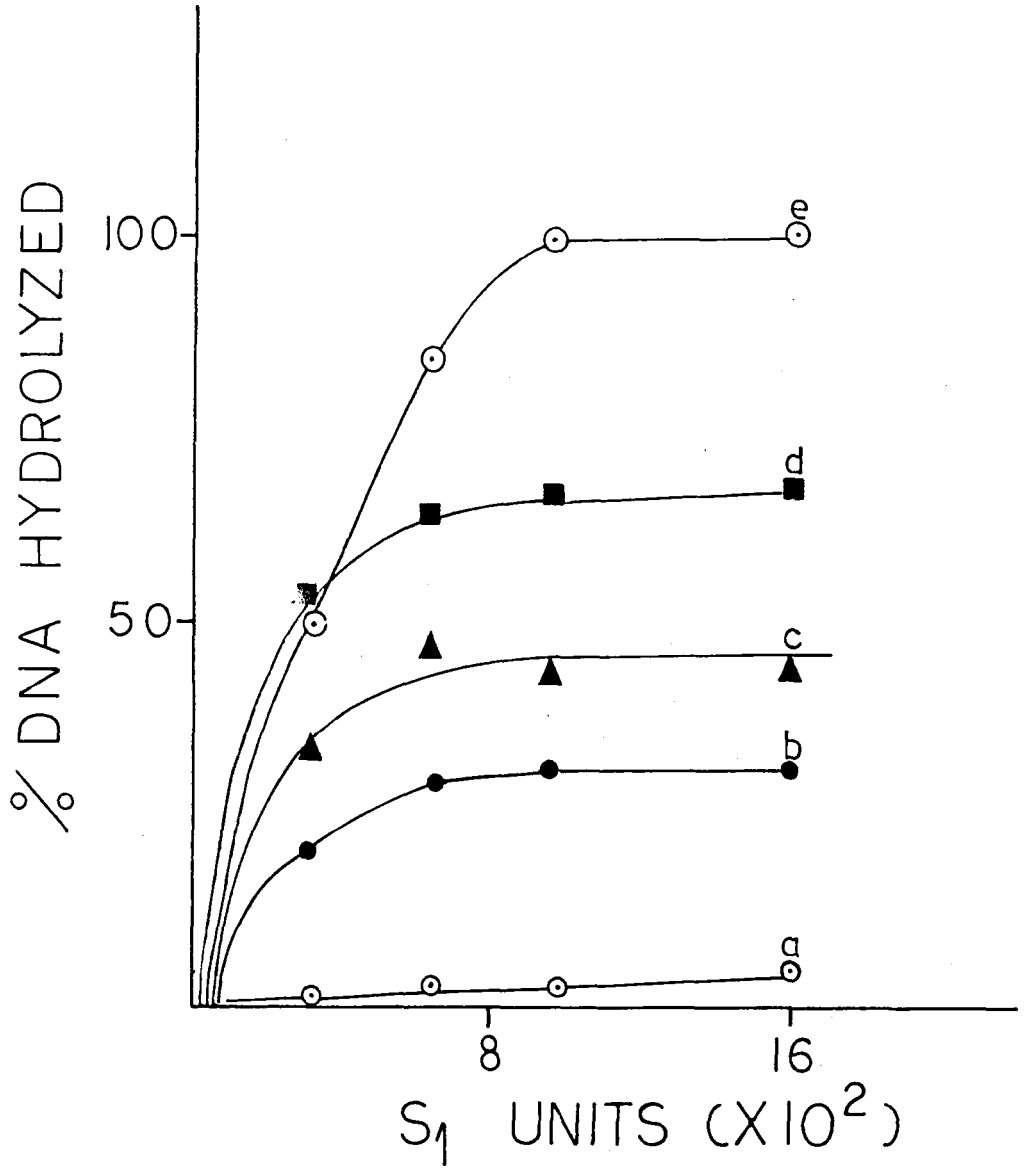
EFFECT OF ALKYLATION ON THE SECONDARY STRUCTURE OF DNA

Degradation of alkylated DNA by S_1 nuclease

DNA was alkylated with an increasing molar ratio of dimethyl sulfate and subjected to hydrolysis by the single-strand-specific

Fig. 9 Degradation of alkylated DNA by S_1 nuclease at various DNA nucleotide/DMS molar ratios.

DNA was alkylated as described in the text, and subsequently dialysed against 50 vol. of TNE containing 0.1M NaCl. The reaction mixture in 1 ml contained 500 ug native, alkylated, or denatured DNA, 0.1M acetate buffer, pH 4.5, containing 1mM $ZnSO_4$, and enzyme units as indicated. The incubation was at 40° for 2 hrs. and the reaction stopped by the addition of 0.2 ml of 10 mg/ml bovine serum albumin and 1.0 ml cold 14% PCA. Acid-soluble nucleotides were measured by the diphenylamine procedure (Schneider, 1957). (a) Native DNA; (b,c,d) DNA alkylated with DNA nucleotide/DMS molar ratio of 1:1, 1:2 and 1:4, respectively; (e) denatured DNA.

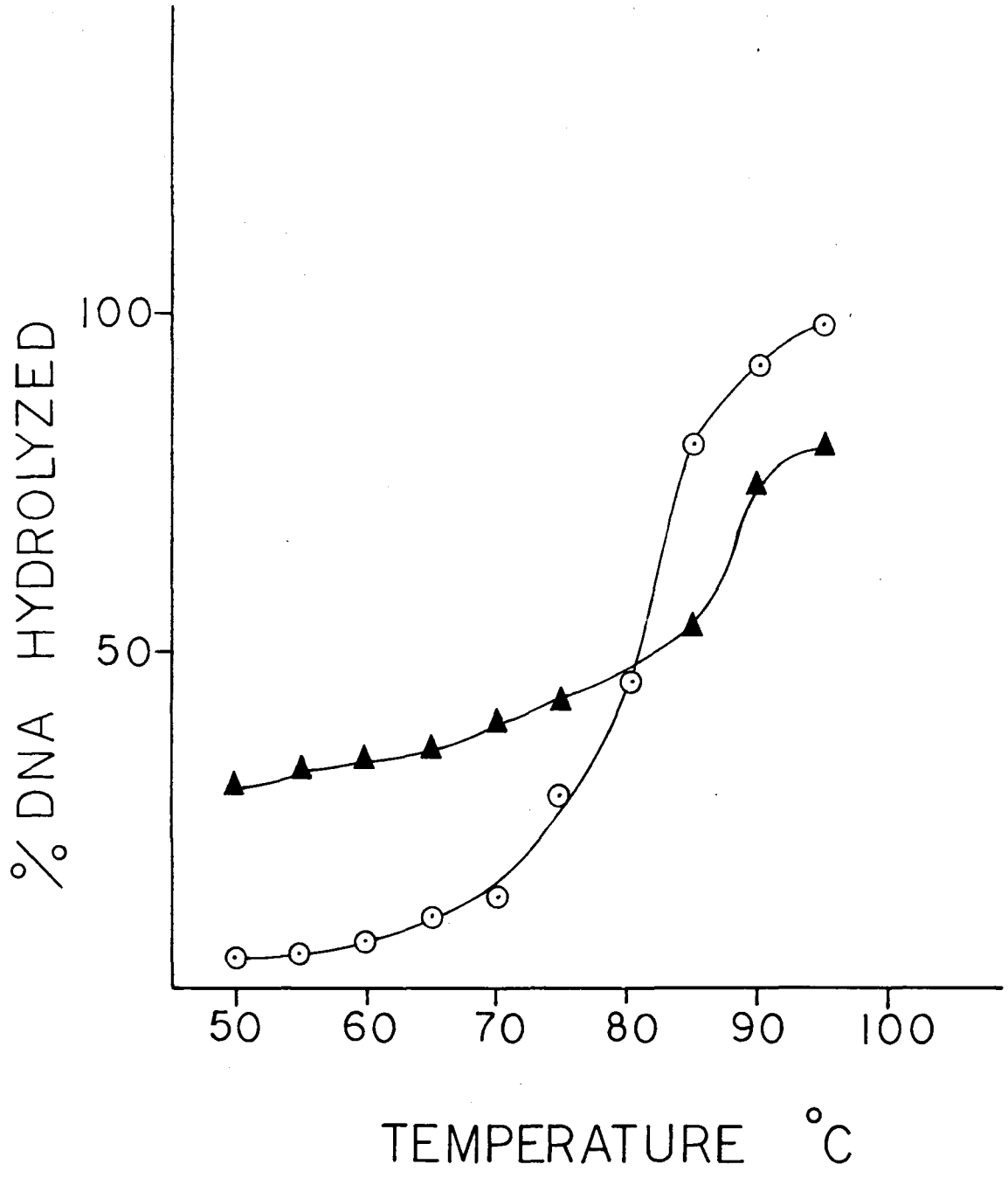

% DNA HYDROLYZED
100
50
e
d
c
b
a
8
16
S1 UNITS (X10²)

nuclease S_1. The results given in Fig. 9 show that with increasing enzyme concentration, production of maximum acid-soluble material increased with DMS molar ratio used for alkylation. Controls using native and denatured DNA showed 4% and 100% degradation respectively. Thus alkylation transforms DNA into an effective substrate for S_1 nuclease possibly due to the formation of partially denatured regions in the native molecule. As already mentioned, alkylation of DNA may lead to denaturation even in the absence of significant depurination. This was considered to be due to a destabilization of the secondary structure caused by an accumulation of positive charges on opposite strands (Wani et al., 1978).

S_1 nuclease has been utilized in studying the secondary structure of DNA through a variety of approaches; e.g., it has been used to determine the thermal melting profile through digestion of the denatured DNA strands. Fig. 10 shows such an experiment with calf thymus DNA alkylated at a DNA nucleotide/DMS molar ratio of 1:1. The mid-range melting temperature (Tm) of control native DNA was determined to be 81°C. The melting profile of alkylated DNA is somewhat unusual since even at temperatures below 60°C considerable hydrolysis of DNA is observed. This is presumably because of depurination of alkylated bases at these temperatures (Verly et al., 1973) which would result in partial denaturation of the double-stranded molecule, rendering these regions digestible by S_1 nuclease. Also,

Fig. 10 Thermal denaturation of native and alkylated DNA as measured by the degree of S_1 nuclease digestion.

DNA was alkylated as described in the text and dialysed against 0.1M NaCl TNE. Samples containing 300 ug DNA were heated to the desired temperature for 8 minutes and then quickly quenched by the addition of 2 vol. of ice-cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 600 units of S_1 nuclease at 40° for 2 hrs. The reaction was terminated and processed as described in Figure 1. Native DNA (O-O); alkylated DNA, nucleotide/DMS ratio 1:1 (▲—▲).


% DNA HYDROLYZED
100
50
50
60
70
80
90
100
TEMPERATURE °C

complete hydrolysis of alkylated DNA is not observed even at 95°C. This may be due to the formation of a limited number of interstrand crosslinks originating from the exposed aldehyde function at the apurinic sites (Burnotte and Verly, 1972). At higher DMS molar ratios progressively decreasing hydrolysis at 95°C was seen, thereby supporting the above idea.

BND-cellulose chromatography of alkylated DNA

BND-cellulose has been used to measure growing points in replicating DNA (Scudiero and Strauss, 1974) and to detect interruptions in human cellular DNA treated with methylmethane sulfonate, an alkylating agent (Karran et al., 1977). Adherence of DNA to BND-cellulose, represented by the fraction which elutes with 2.0 % caffeine in 1M NET, is considered to be due to two properties: binding of single-stranded regions, or binding of aromatic residues to the resin (Karran et al., 1977). In Figure 11 b,c,d, and e is shown the BND-cellulose chromatography of DNA samples alkylated with increasing molar ratios of DMS in 0.1M NaCl TNE. DNA without single-stranded breaks or regions, obtained by prechromatography of calf thymus DNA on BND-cellulose, was used in these experiments. Increasing number of DNA molecules show adherence to BND-cellulose with a concomitant decrease in the material eluting with 1M NET. The DNA sample alkylated at a DNA nucleotide/DMS molar ratio of 1:6, where almost 80% molecules adhere to BND-cellulose, was also

Fig. 11 Chromatography of alkylated DNA on BND-cellulose under various conditions.

To obtain DNA without single-stranded breaks or regions, calf thymus DNA was prechromatographed on BND-cellulose. The 1M NET eluate was dialysed against 0.01M or 0.1M NaCl TNE. Control DNA was also dialysed. 100 ug DNA samples were alkylated for 1 hr with sufficient dimethyl sulfate (diluted 10-fold in TNE) to obtain the desired molar ratio. The alkylated DNA was directly applied to the column (1 x 2.5 cm) and step-eluted with 0.3M NET, 1M NET, and 1M NET plus 2.0% caffeine. 2 ml fractions were collected at the rate of 8ml/hr. DNA was measured by the diphenylamine method of Burton (1956). Recoveries in all experiments ranged between 80-100%. (a) Control; (b,c,d,e) DNA alkylated in 0.1M NaCl with the DNA nucleotide/DMS molar ratio of 1:2, 1:4, 1:6 and 1:8 respectively; (f) same as d but treated with S1 nuclease after alkylation; (g) control DNA in 0.01M NaCl TNE; (h) DNA in 0.01M NaCl TNE alkylated with a nucleotide/DMS ratio of 1:2.

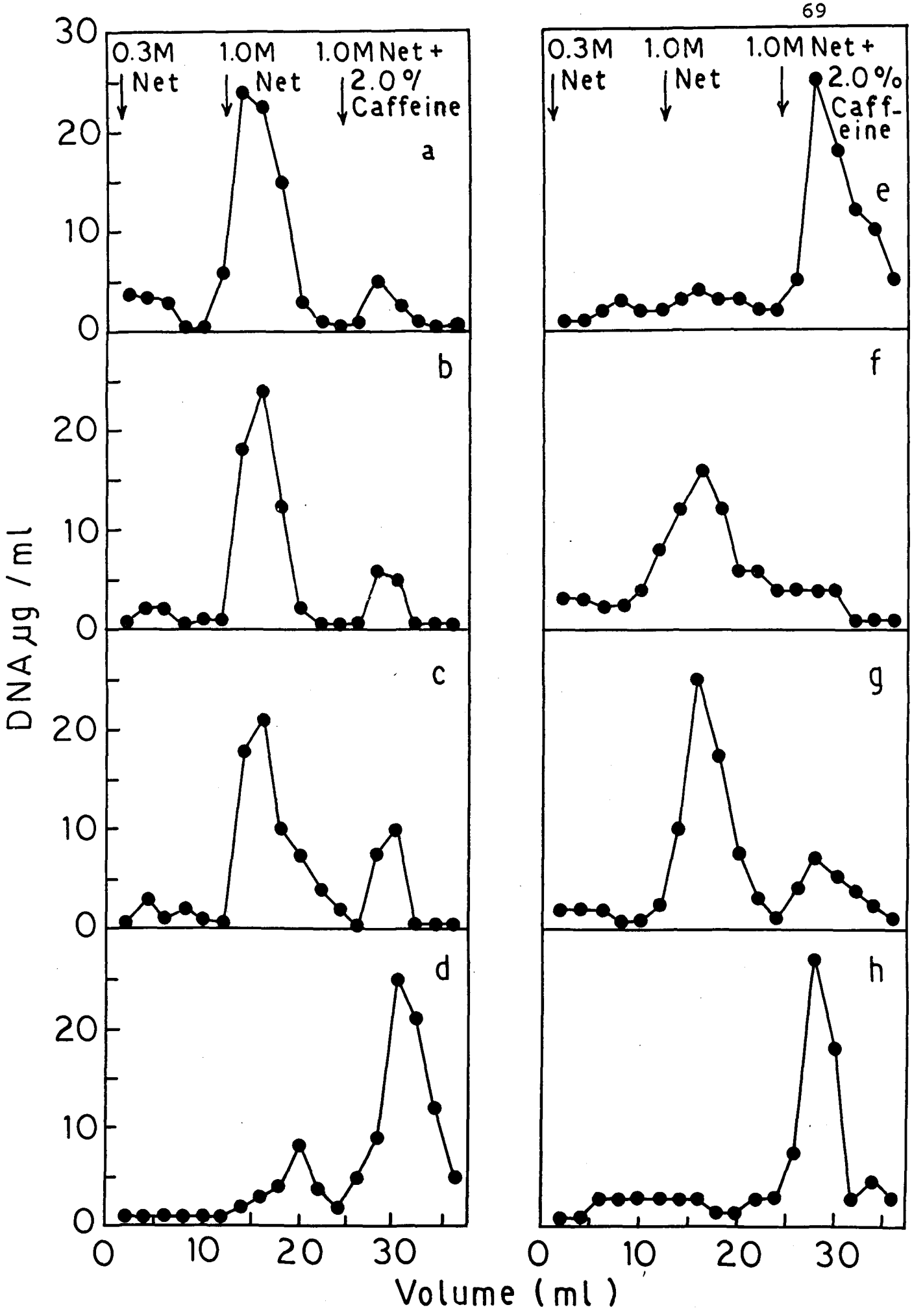
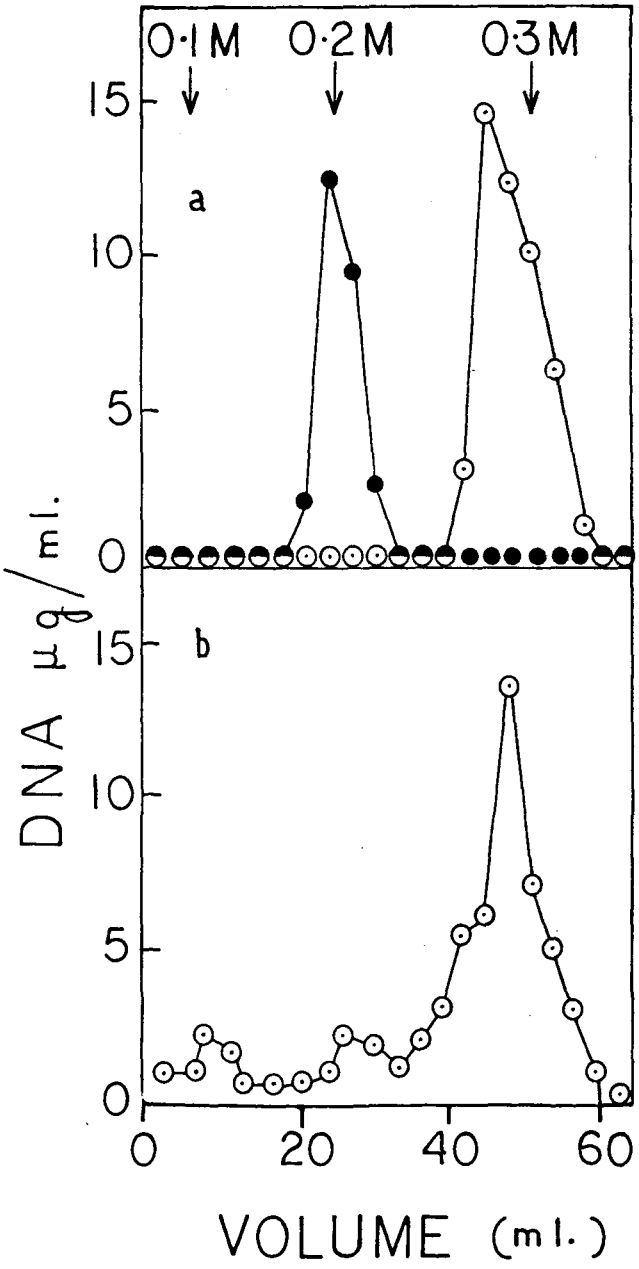

0.3M Net
1.0M Net
1.0M Net + 2.0% Caffeine
0.3M Net
1.0M Net
1.0M Net + 2.0% Caff-eine
a
b
c
d
e
f
g
h
DNA µg / ml
Volume (ml)
0
10
20
30

Fig. 12 Hydroxyapatite chromatography of DNA alkylated with a nucleotide/DMS molar ratio of 1:6 in 0.1M NaCl TNE.

DNA without single-stranded regions was obtained by pre-chromatography on BND-cellulose, and a 200 ug sample was alkylated as described in the text. It was loaded on a 1x3 cm column, previously equilibrated with 0.01M sodium phosphate buffer, pH 7.0, and immediately followed by elution with sodium phosphate buffer (pH 7.0) of indicated molarities. 3-ml fractions were collected at the rate of 10 ml/hr. Recovery was approximately 78%. (a) Native DNA (0-0), denatured DNA (0-0); (b) alkylated DNA.


0·1M
0·2M
0·3M
a
b
15
10
5
0
15
10
5
0
DNA µg/ml.
0
20
40
60
VOLUME (ml.)

chromatographed on hydroxyapatite (Fig. 12). Almost all the DNA eluted at the position of double-stranded DNA suggesting that adherence to BND-cellulose is not the result of complete denaturation of DNA molecules. Alkaline hydrolysis of alkylated DNA samples used in these experiments showed no production of acid-soluble nucleotides, suggesting that no significant depurination of alkylated sites had occurred. In Fig. 11f the DNA was alkylated at a molar ratio of 1:6, and subsequently treated with S_1 nuclease before applying to BND-cellulose. As expected, the remaining DNA after digestion with the nuclease does not adhere to the resin, and elutes with 1M NET. It is therefore concluded that alkylation by DMS under our conditions causes the formation of partially denatured DNA molecules. When DNA was alkylated in 0.01M NaCl TNE at a DNA nucleotide/DMS molar ratio of 1:2, 78% of the molecules adhere to BND-cellulose (Fig. 11h) compared with 18% in the presence of 0.1M NaCl TNE (Fig. 11b). A control DNA sample kept in 0.01M NaCl TNE showed only 25% adherence.

DISCUSSION

Monofunctional alkylating agents alkylate DNA at two sites: bases and phosphates. However, the major sites of alkylation with DMS are the N-7 position of guanine and N-3 position of

adenine, and it causes minimum alkylation of DNA phosphates (Lawley and Thacher, 1970). Our results suggest that alkylation of DNA may lead to the formation of partially denatured molecules even in the absence of depurination of alkylated bases. A possible mechanism could be that the positive charges of the quaternized alkylated bases through repulsion may adversely affect the forces stabilizing the secondary structure. This is strengthened by the observation that the presence of salt has a preventive effect on the formation of partially denatured molecules. Hsiung et al., (1976) have postulated that partial denaturation of alkylated DNA at higher pH values may occur through the disruption of hydrogen bonding due to a base-catalyzed imidazole ring opening of the quaternized N-7 guanine. There is now considerable evidence that changes in DNA conformation, such as distortions in the secondary structure produced by intercalating agents or regions of local denaturation, are recognized by DNA repair or other nucleases producing strand breakage (Duncan et al., 1976; Warren et al., 1979). Indeed, it has been suggested that the cytotoxic effect of many drugs may be better related to DNA damage as a result of the action of nucleases that recognize distortion of the helical structure, rather than to the physical presence of the bound drug itself (Waring, 1981).

USE OF S_1 NUCLEASE IN PROBING THE SECONDARY STRUCTURE OF DNA COMPLEXED WITH INTERCALATING AND NON INTERCALATING AGENTS

Action of S_1 nuclease on complexes of DNA with intercalating and non intercalating agents

S_1 nuclease from Aspergillus oryzae has proved to be extremely useful in studying the secondary structure of DNA. It recognizes distortions in base pairing caused by deletions and damage by chemicals and ultraviolet light (Silber and Loeb, 1981; Shishido and Ando, 1974; Weigand et al., 1975). Intercalating agents are a cytotoxic group of drugs which reversibly bind to DNA by intercalation between adjacent base pairs (Fuller and Waring, 1964). In the following experiments I have used S_1 nuclease to explore the changes in the secondary structure of DNA complexed with intercalating and non intercalating agents. Figure 13 shows the degradation of native calf thymus DNA complexed with increasing relative concentrations of ethidium bromide. DNA was first treated with ethidium bromide before the addition of S_1 nuclease. A higher temperature of 48°C was used for enzyme incubation in order to expose the less stable secondary structures in DNA to the enzyme. Using denatured DNA it was earlier shown that S_1 nuclease is not inhibited at the concentrations of ethidium bromide tested (Table V). A progressive decrease in the rate of DNA hydrolysis was observed with increasing relative concentrations of ethidium bromide (Figure 13).

Fig. 13 Effect of S_1 nuclease on native DNA and ethidium bromide - DNA complex

2.1 mg of native calf thymus DNA in 1.5 ml TNE was preincubated at room temperature for 1 hour with varying amounts of ethidium bromide to give the desired DNA bp/drug molar ratios. After the preincubation period, stock S_1 nuclease buffer, glycerol to a final concentration of 20% and 4800 units of S_1 nuclease were added and the volume made up to 7.0 ml. Incubation was carried out at 48°C. Aliquots containing 300 ug substrate DNA were removed at time intervals indicated and added to tubes containing 0.2 ml of 2 mg/ml BSA and 1 ml ice cold 14% PCA. The tubes were stored at 4°C for 1 hour before centrifugation. The acid soluble nucleotides were determined by the diphenylamine procedure (Schneider, 1957).

Native DNA (O——O)

DNA bp/EtBr molar ratio 5:1 (●——●)

DNA bp/EtBr molar ratio 10:1 (△——△)

DNA bp/EtBr molar ratio 20:1 (▲——▲)

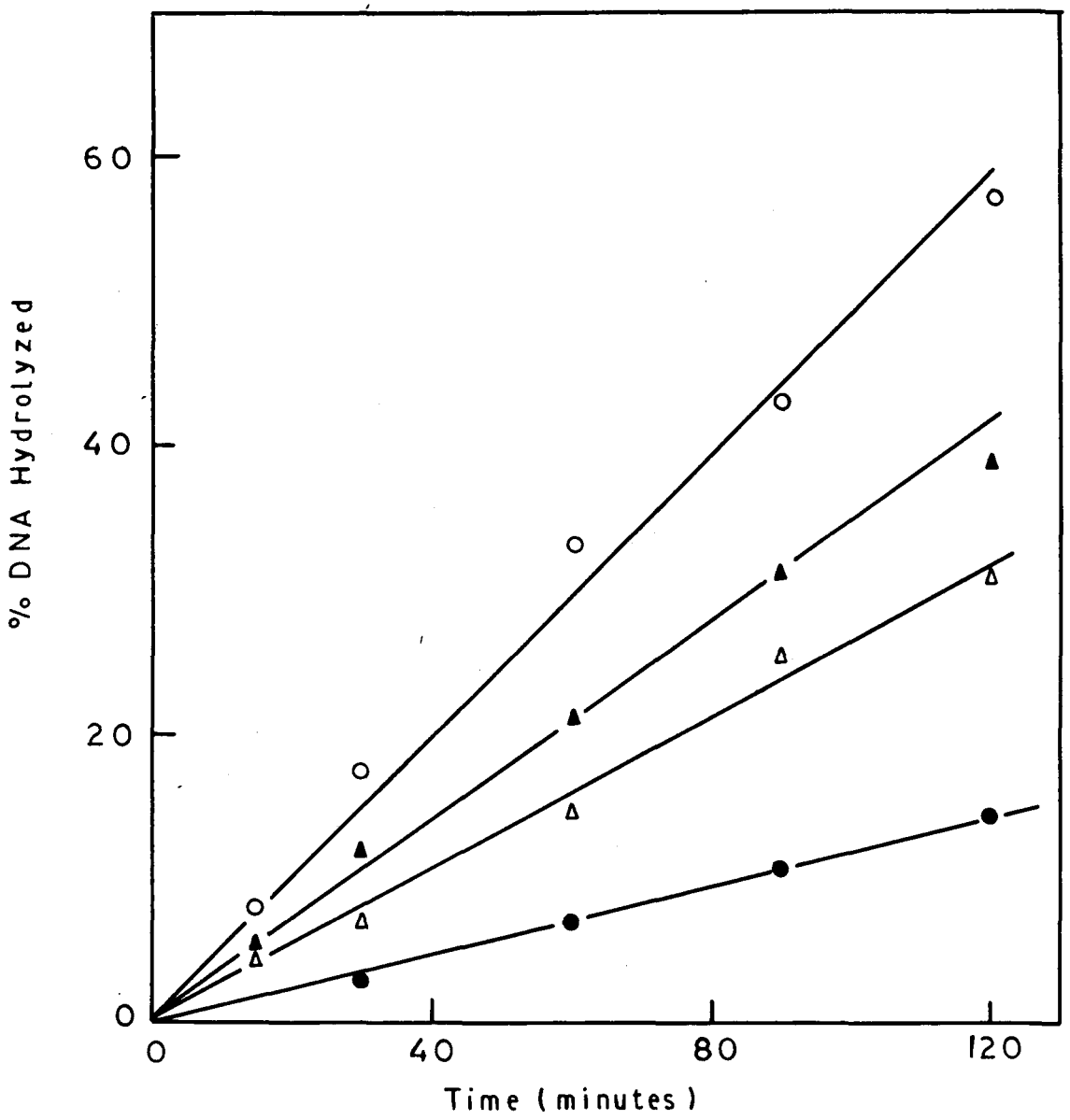

% DNA Hydrolyzed
60
40
20
0
0
40
80
120
Time (minutes)

Table V

EFFECT OF INCREASING CONCENTRATION OF INTERCALATING AND NON-INTERCALATING AGENTS ON S_1 NUCLEASE ACTIVITY

DNA base pair/ Drug molar ratio	Denatured DNA hydrolysis(% of control)			
	Ethidium bromide	Acridine orange	Spermine	Streptomycin sulfate
CONTROL (no drug)	100.00	100.00	100.0	100.0
20:1	100.0	100.0	100.0	100.0
10:1	97.0	100.0	106.2	100.0
5:1	91.0	100.0	100.0	98.3
3:1	81.0	92.0	100.0	102.2
2:1	60.0	87.0	100.0	98.3

Appropriate amounts of drug solutions (in water) were added to 300 ug of heat denatured DNA in 0.3 ml TNE to achieve the desired DNA bp/drug molar ratios. The reaction mixture in a total of 1 ml also contained 0.1M acetate buffer pH 4.5 and 1mM $ZnSO_4$. Incubation was performed with 800 units of S_1 nuclease at 40°C for 2 hours. The reaction was terminated and processed as described in 'Methods'.

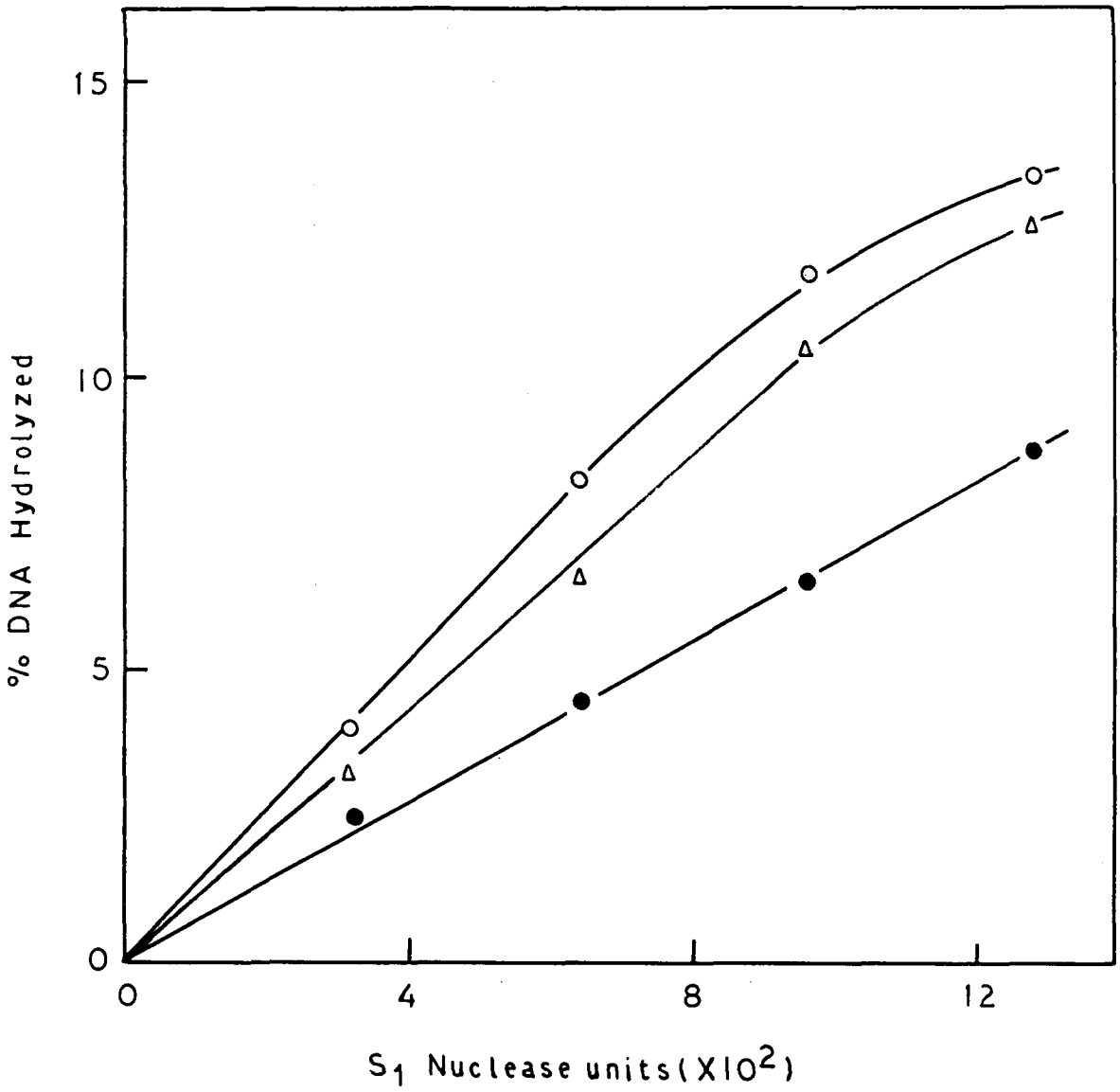
Fig. 14 Degradation by S_1 nuclease of DNA complexed with ethidium bromide and spermine.

To 600 µg DNA in 0.3 ml TNE was separately added 65 ug ethidium bromide and 20 ug spermine (in TNE) to achieve a DNA bp/drug molar ratio of 6:1. The reaction mixture in 1 ml also contained 50 mM acetate buffer pH 4.5, 0.5 mM $ZnSO_4$ and 20% glycerol (final concentration), water and enzyme units as indicated. The incubation was carried out at 48°C for 2 hours and the reaction stopped by the addition of 0.2 ml of 2 mg/ml BSA and 1 ml cold 14% PCA. Further processing for the determination of acid soluble nucleotides was done as described in 'Methods'.

Native DNA (○——○)

DNA bp/ EtBr molar ratio 6:1 (●——●)

DNA bp/spermine molar ratio 6:1 (Δ——Δ)


15
10
5
0
% DNA Hydrolyzed
0
4
8
12
S1 Nuclease units (X10²)

In figure 14, with increasing enzyme concentration, the production of maximum acid soluble material was studied using DNA complexed with ethidium bromide and spermine, which binds to DNA by non intercalative means. The decrease in maximum DNA hydrolyzed is considerably greater in the case of ethidium bromide than with spermine. The above observations are therefore in agreement with the property of intercalating agents of stabilizing the secondary structure of DNA (Lerman, 1964).

S_1 nuclease has also been used to determine the thermal melting profile of native DNA through digestion of denatured DNA strands (Case and Baker, 1975). Figure 15 shows such an experiment with native DNA complexed with various molar ratios of ethidium bromide. The mid range melting temperature of control native DNA was determined to be 73°C. The melting profile of DNA-ethidium bromide complex was found to be biphasic at DNA bp/ethidium bromide molar ratios of 20:1 and 10:1. At the higher ratio of 5:1 hardly any denaturation of DNA was achieved. Also, complete hydrolysis of DNA was not observed even at 95°C and progressively decreasing maximum hydrolysis was seen. Similar biphasic melting profiles are also observed with another known intercalating agent, acridine orange (Fig. 16). However, in this case, with similar DNA bp/drug molar ratios the DNA duplex is not stabilized to the same extent as with ethidium bromide since considerable denaturation of DNA is achieved even with the highest acridine orange molar ratio tested. Also, the biphasic

Fig. 15 Thermal melting profile of native and ethidium bromide (EtBr) treated DNA at various DNA bp/EtBr molar ratios as determined by the degree of S_1 nuclease digestion.

300 µg DNA in 0.15 ml TNE was treated with varying amounts of ethidium bromide to achieve the desired DNA bp/EtBr molar ratios. The samples were heated to the desired temperature for 8 minutes and then quickly quenched by the addition of 2 volumes of ice cold S_1 nuclease stock reaction buffer. The mixture was then incubated with 800 units of S_1 nuclease at 40°C for 3 hours. The reaction was terminated and processed as described in 'Methods'.

Native DNA (○——○)

DNA bp/EtBr molar ratio 20:1 (●——●)

DNA bp/EtBr molar ratio 10:1 (▲——▲)

DNA bp/EtBr molar ratio 5:1 (△——△)

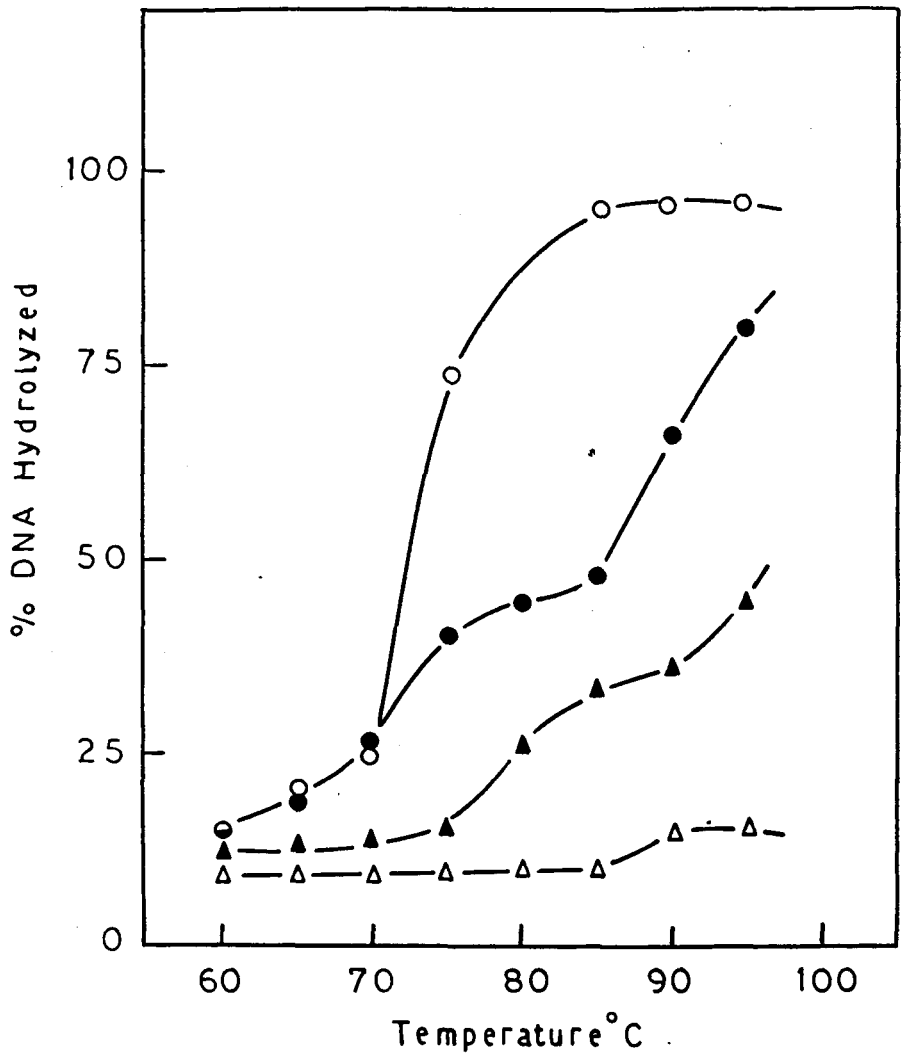

% DNA Hydrolyzed
100
75
50
25
0
60
70
80
90
100
Temperature °C

Fig. 16 Thermal melting profile of native and acridine orange (AO) treated DNA at various DNA bp/AO molar ratios as determined by the degree of S_1 nuclease digestion.

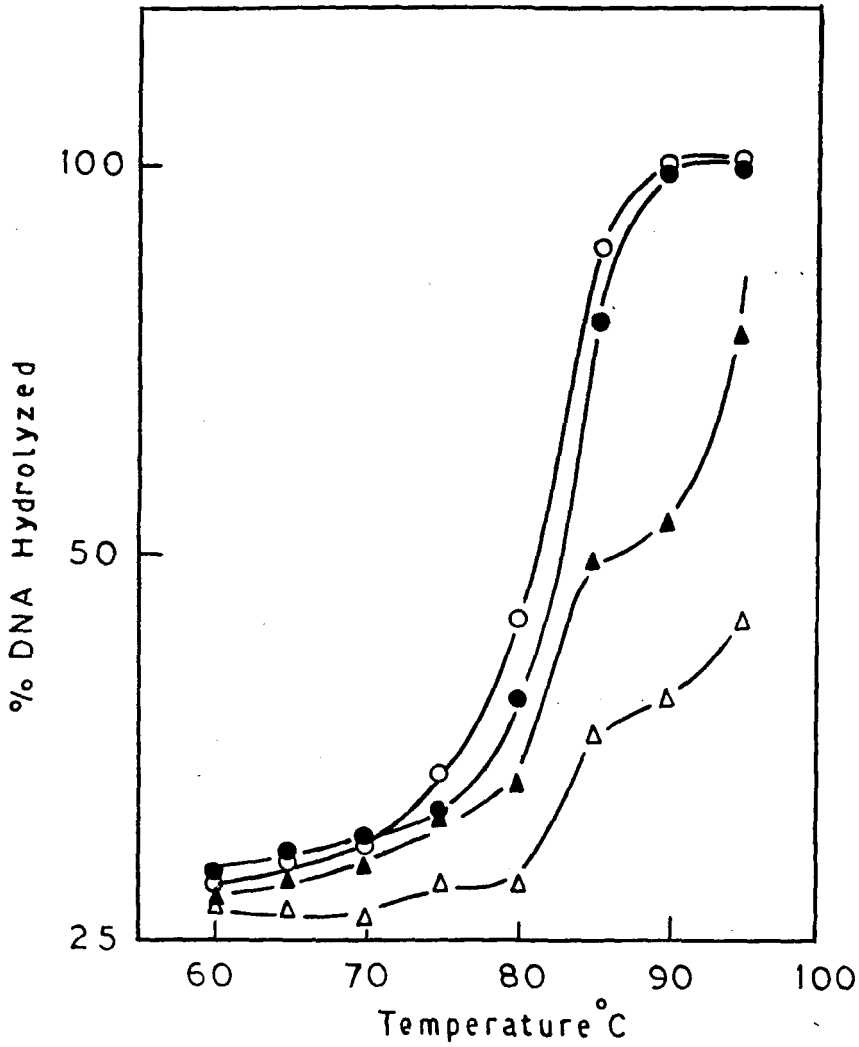
300 µg DNA in 0.15 ml TNE was treated with varying amounts of acridine orange to obtain the desired DNA bp/AO molar ratios. Each sample was heated to the desired temperature for 8 minutes and then quickly quenched by addition of 2 volumes of ice cold S_1 nuclease stock reaction buffer. The mixture was then incubated with 800 units of S_1 nuclease at 40°C for 3 hours and was terminated processed as described in 'Methods'.

Native DNA (○——○)

DNA bp/AO molar ratio 10:1 (●——●)

DNA bp/AO molar ratio 5:1 (△——△)

DNA bp/AO molar ratio 2.5:1 (▲——▲)


100
50
25
% DNA Hydrolyzed
60
70
80
90
100
Temperature °C

nature of the melting profile is observed only at DNA bp/acridine orange molar ratios of 5:1 and 2.5:1. DNA melting profiles were also determined with streptomycin sulfate and spermine, two ligands which bind to DNA by non intercalative surface interactions (Fig. 17). Although an increase in melting temperature with reduced maximum hydrolysis is observed, the biphasic nature of the profile was absent.

Activity of S_1 nuclease on alkylated DNA in the presence of of denaturing solvents

Single strand specific nucleases are a useful tool for studying low melting regions in DNA. The convenience and usefulness of such enzymes can be further enhanced if they were shown to be active in solvents which destabilize the DNA duplex. This would increase the range of experimental conditions under which the secondary structure of nucleic acids can be explored. S_1 nuclease has been shown to be active in the presence of various concentrations of solvents such as formamide, dimethylsulfoxide, formaldehyde and glyoxal (Case and Baker, 1975; Hutton and Wetmur, 1975). We have studied the activity of S_1 nuclease on alkylated and depurinated DNA in the presence of dimethylsulfoxide and formamide. As seen in Fig. 18 the enzyme is about 50% less active on DNA alkylated at the DNA nucleotide/dimethyl sulfate molar ratio of 1:4. When depurinated DNA obtained from such alkylated DNA was used as the substrate the

Fig. 17 Thermal denaturation of native, streptomycin sulfate and spermine treated DNA as measured by the degree of S_1 nuclease digestion.

Sufficient streptomycin sulfate and spermine were added to 300 µg DNA samples in 0.15 ml TNE to obtain a DNA bp/drug molar ratio of 5:1. The samples were heated to the desired temperature for 8 minutes and then quickly quenched by addition of 2 volumes of ice cold S_1 nuclease stock reaction buffer. Incubation was carried out with 800 units of S_1 nuclease at 40°C for 3 hours. The reaction was terminated and processed as described in 'Methods'.

Native DNA (●—●)

DNA bp/strep. Sulfate molar ratio 5:1 (▲—▲)

DNA bp/spermine molar ratio 5:1 (○—○)

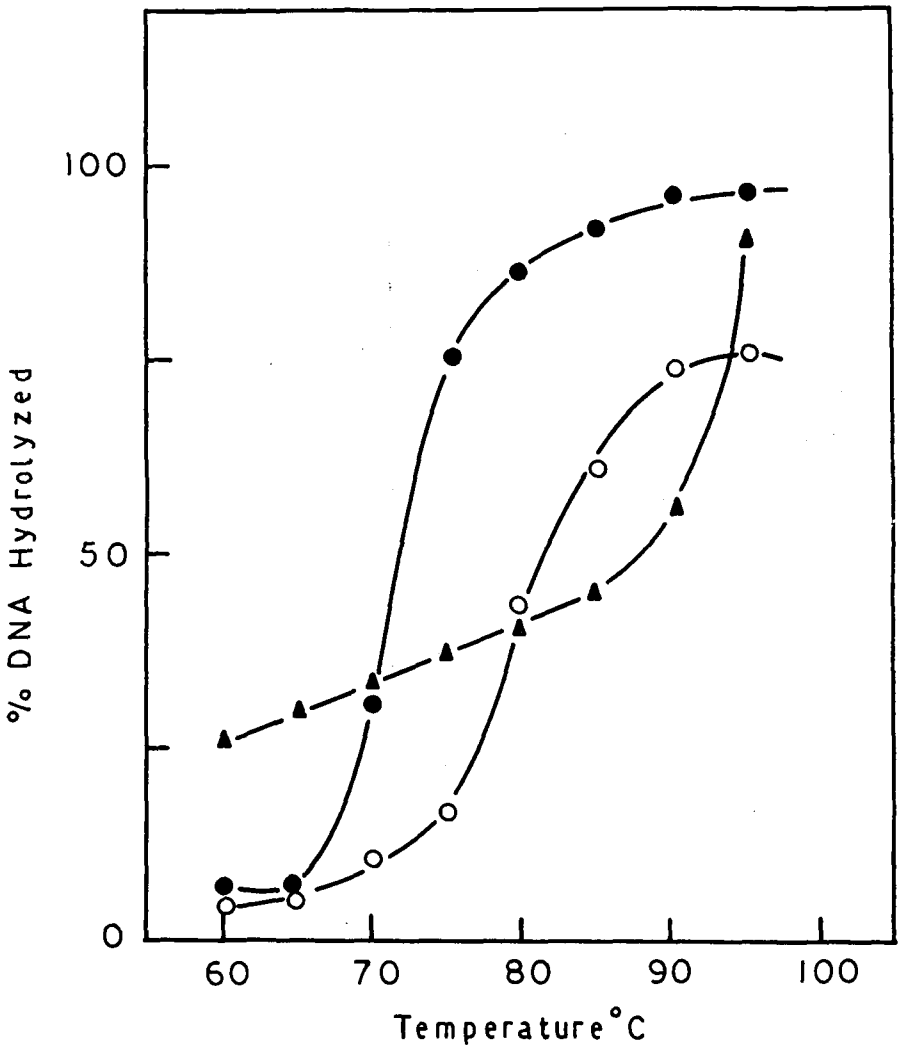

100
50
0
% DNA Hydrolyzed
60
70
80
90
100
Temperature °C

Fig. 18 Effect of S_1 nuclease on denatured, alkylated and depurinated DNA.

Denatured DNA was prepared by heating a 1-2 mg/ml solution of DNA in TNE at 100°C for 7 minutes followed by rapid cooling in ice. For preparing alkylated DNA, a 1-2 mg/ml solution of DNA in TNE was methylated by adding sufficient dimethyl sulfate (DMS) to obtain a DNA nucleotide/DMS molar ratio of 1:4. The solution was gently shaken at 25°C for 1 hour. The acid released by the hydrolysis of DMS was neutralized by the addition of enough 0.1M NaOH and pH was maintained between 6 and 7. Depurinated DNA was obtained by heating alkylated DNA at 50°C for 4 hours. To 1.5 mg of substrate DNA (denatured, alkylated and depurinated) in 2 ml TNE, standard S_1 nuclease reaction buffer and 4000 units of S_1 nuclease were added and the volume made up to 5.0 ml with water. Incubation was carried out at 37°C. 1 ml aliquots containing 300 ug substrate DNA were removed at time intervals indicated and the reaction terminated and processed as described in 'Methods'.

Heat denatured DNA (△——△)

Depurinated DNA (●——●)

Alkylated DNA (○——○)

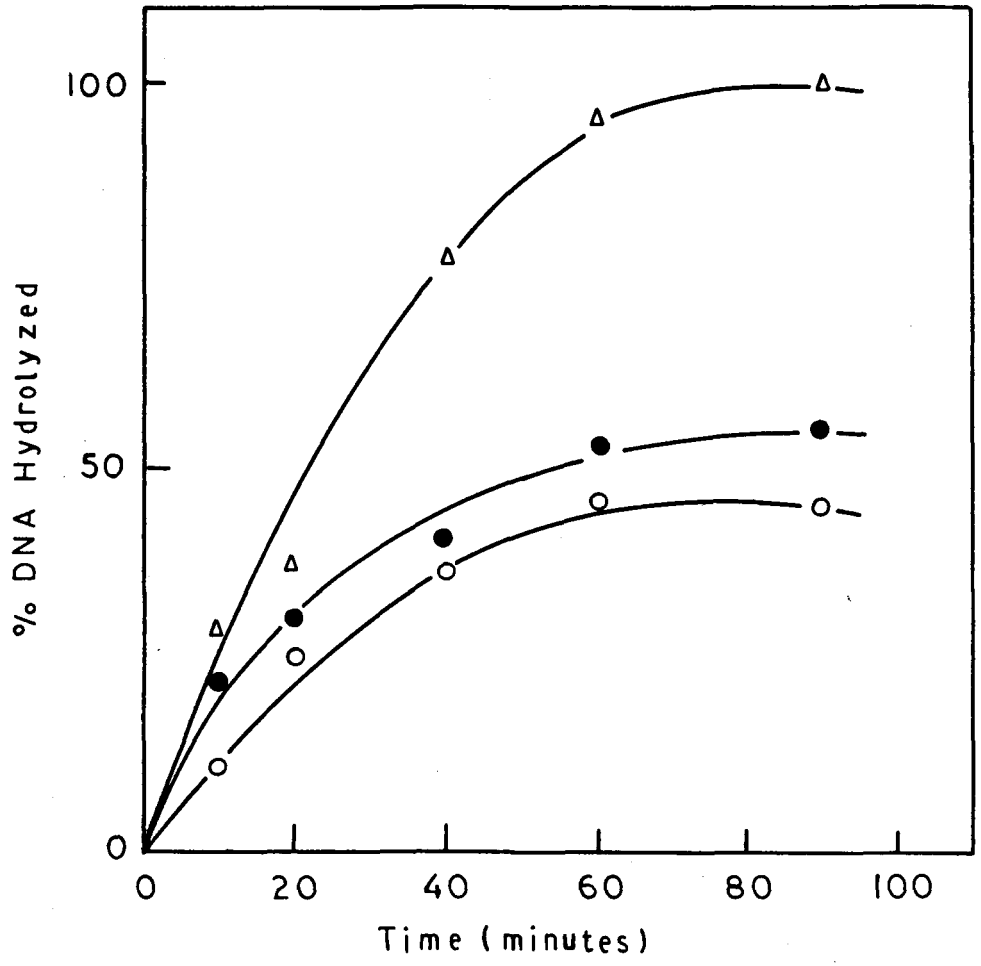

100
50
0
% DNA Hydrolyzed
0
20
40
60
80
100
Time (minutes)

Fig. 19 **Effect of S_1 nuclease on alkylated DNA in presence of dimethylsulfoxide and formamide.**

Alkylated DNA was prepared as described in figure 18. To 1.5 mg alkylated DNA was added stock S_1 nuclease reaction buffer, dimethylsulfoxide and formamide to a final concentration of 5%, 3200 units of S_1 nuclease and the volume made upto 5.0 ml. Incubation was carried out at 37°C and 1 ml aliquots containing 300 ug substrate DNA were drawn at time intervals indicated. The reaction was terminated and processed as described in 'Methods'.

Alkylated DNA (alone) (○——○)

Alkylated DNA in presence of 5% dimethylsulfoxide (●——●)

Alkylated DNA in presence of 5% formamide (△——△)

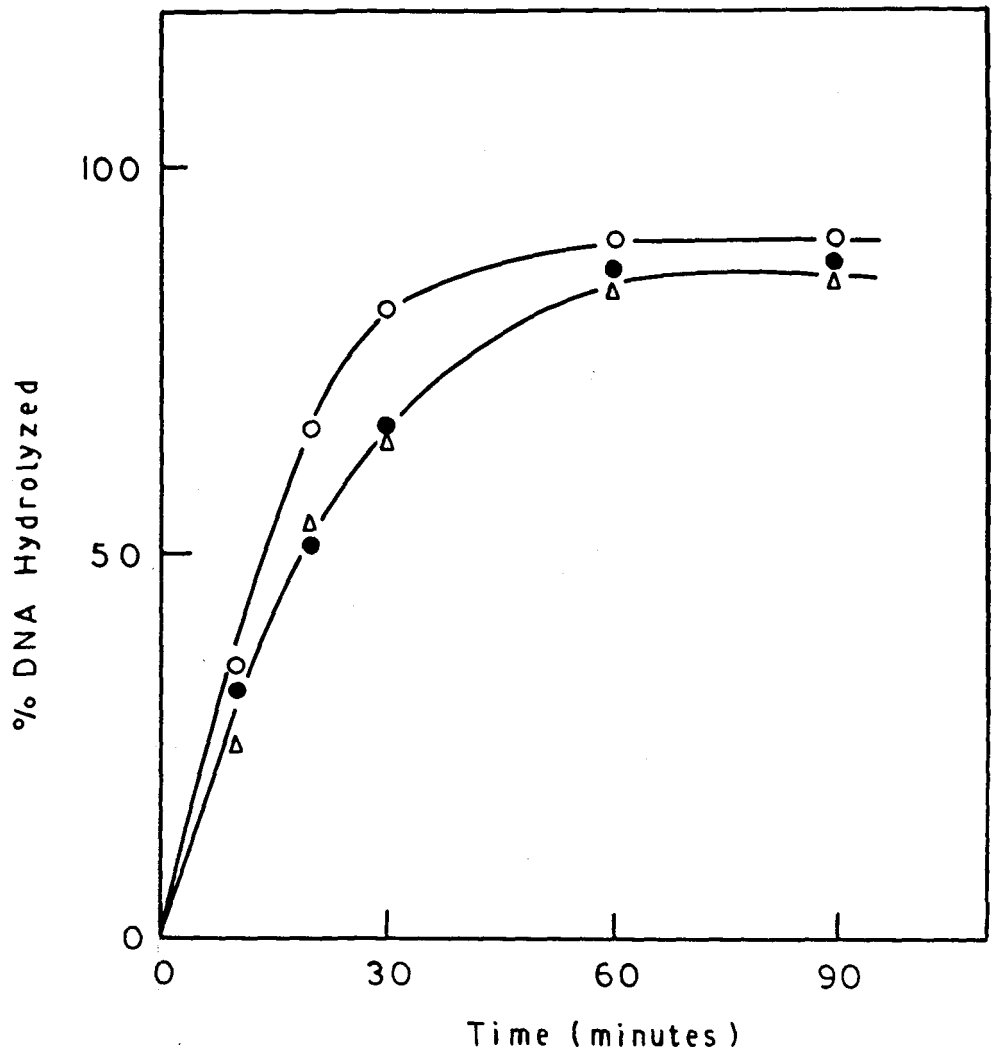

100
50
0
% DNA Hydrolyzed
0
30
60
90
Time (minutes)

Table VI

EFFECT OF INCREASING SODIUM CHLORIDE CONCENTRATION ON THE DEGRADATION OF DENATURED, ALKYLATED AND DEPURINATED DNA BY S_1 NUCLEASE

Concentration of sodium chloride (M)	% DNA Hydrolysed		
	Denatured DNA	Alkylated DNA DNA nucleotide/ DMS Molar ratio = 1:2	Depurinated DNA
NONE (Control)	100.0	100.0	100.0
0.05	100.0	100.0	100.0
0.10	100.0	92.0	95.0
0.15	100.0	90.0	91.0
0.20	84.0	86.0	78.0
0.25	72.0	76.0	67.0

Alkylated and depurinated DNAs were prepared as described in figures 18 and 20 respectively. The reaction mixture in 1 ml contained 400 ug DNA (denatured, alkylated and depurinated) 50 mM acetate buffer, 0.5 mM $ZnSO_4$ and indicated final molarities of sodium chloride. The incubation was started after addition of 800 units of S_1 nuclease at 40°C for 2 hours. The reaction was terminated and processed as described in 'Methods'.

reduced activity of the enzyme was maintained. Fig. 19 shows a similar experiment with alkylated DNA in the presence of 5% dimethylsulfoxide and formamide. No inhibition of enzyme activity was seen. Presence of increasing concentration of sodium chloride effects the hydrolysis of denatured, alkylated and depurinated DNA by S_1 nuclease to about the same degree. Depurinated DNA was also prepared by heating native DNA at acidic pH at various temperatures. Treatment at increasing temperatures leads to increased depurination causing increasing destabilization of the secondary structure (Hadi and Goldthwait, 1971). When depurinated DNA prepared at 40°C, 50°C, 60°C and 70°C was hydrolyzed with S_1 nuclease an increasing degree of maximum hydrolysis was observed as expected (Fig. 20).

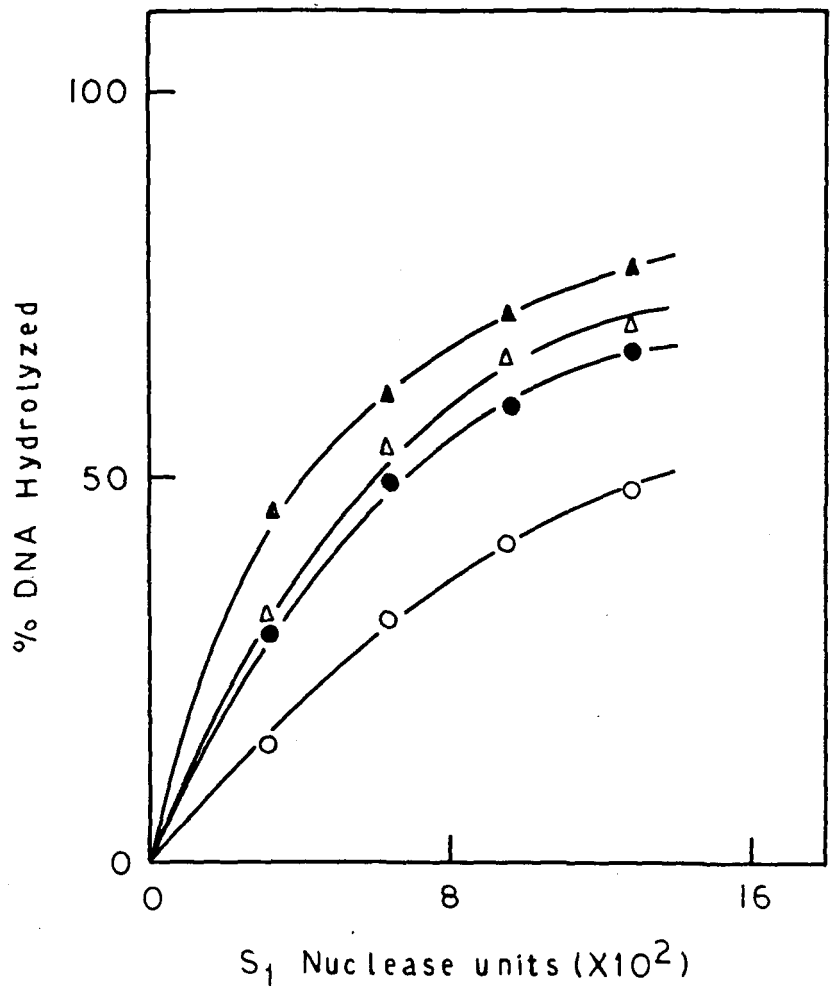
DISCUSSION

Nucleases specific for single stranded DNA or for duplex DNA containing single stranded or weakly hydrogen bonded regions are valuable tools in probing the secondary structure of nucleic acids. Of this class of enzymes the nuclease S_1 from *Aspergillus oryzae* is the most extensively characterized and experimentally used enzyme (Wani and Hart, 1981). It has been used to detect damage in duplex DNA by those chemical and physical agents which destabilize the DNA duplex and cause disruptions

Fig. 20 **Degradation by S_1 nuclease of depurinated DNA prepared at 40°C, 50°C, 60°C and 70°C respectively.**

Depurinated DNA was prepared by adding 3 ml of a 2 mg/ml solution of DNA in TNE to 6 ml of 0.1 M citrate buffer pH 3.5. The pH of the solution was thus brought down to 3.5. This was heated for 20 minutes at the indicated temperature and cooled rapidly in ice. Sufficient 2N NaOH was added to bring the pH to 7.0 and the depurinated DNA was stored at 4°C. The reaction mixture in 1 ml contained 400 ug of depurinated DNA, 0.1 M acetate buffer pH 4.5, 1mM $ZnSO_4$ and enzyme units as indicated. Incubation was done at 37°C for 2 hours. The reaction was terminated and processed as described in 'Methods'.

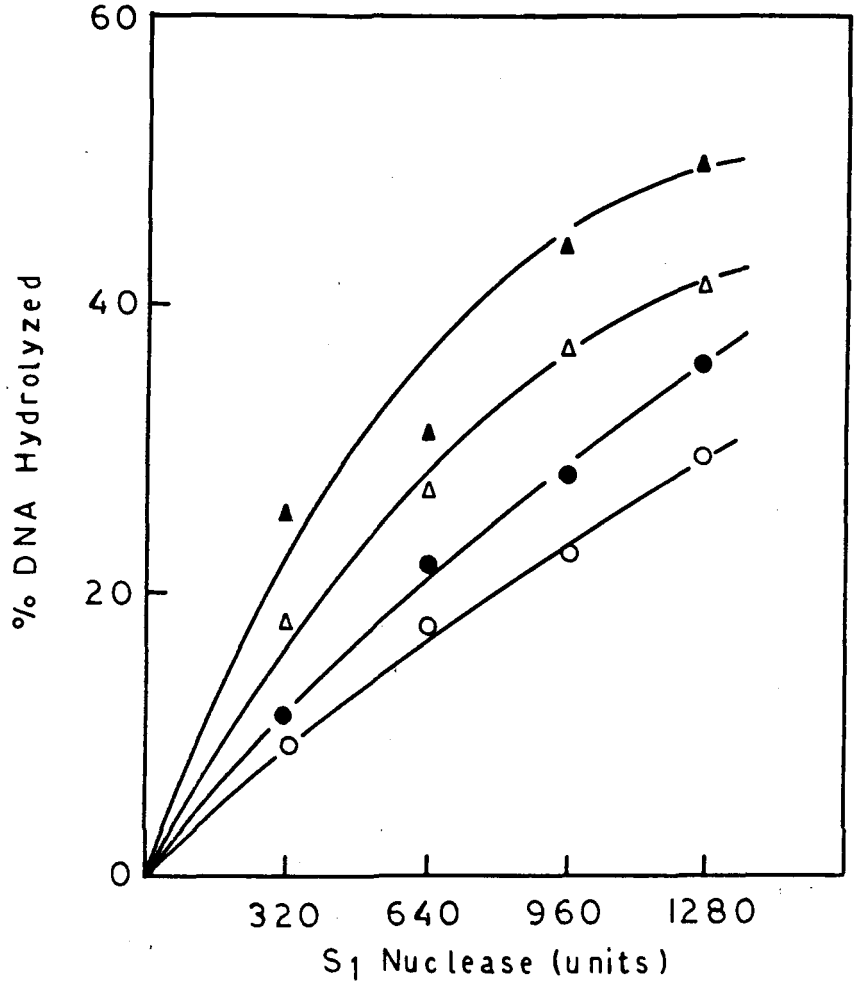
40°C depurinated DNA (○——○)
50°C depurinated DNA (●——●)
60°C depurinated DNA (△——△)
70°C depurinated DNA (▲——▲)


100
50
0
% DNA Hydrolyzed
0
8
16
S1 Nuclease units (X10²)

in hydrogen bonding. However, it can also be used to study the effect of those DNA binding drugs which enhance the stability of the DNA duplex such as intercalating agents (Lerman, 1964). In the above experiments we have determined the thermal melting profiles of DNA complexed with some representative intercalating and non intercalating drugs. An important observation is the biphasic thermal melting profiles obtained with ethidium bromide and acridine orange. It has been reported that a majority of intercalating agents show G-C specificity in DNA binding (Muller and Crothers, 1975) and those that do not, may exhibit a C-G sequence preference in binding (Kastrup et al., 1978; Reinhardt and Krugh, 1978). Possibly this accounts for the biphasic melting profile with ethidium bromide and acridine orange, since at limiting drug concentrations G-C rich regions would be considerably more stabilized than A-T rich regions. At DNA bp/drug molar ratio of 10:1, ethidium bromide shows a considerable stabilization of the DNA duplex whereas no effect is observed with acridine orange. This is presumably indicative of the relative affinities of the two drugs to calf thymus DNA.

Fig. 21 Degradation of riboflavin treated DNA by S_1 nuclease after various periods of incubation in visible light.

A solution (0.5 ml, 0.01M Tris-HCl pH 7.4, 0.01 M NaCl, 2×10^{-4}M (EDTA) containing native calf thymus DNA equivalent to 3mM DNA nucleotide and 0.16 mM riboflavin (DNA bp/riboflavin molar ratio 1:18) was incubated in fluorescent light at 25°C for the time periods indicated. After incubation, S_1 nuclease reaction was started by the addition of 0.1M acetate buffer, 1mM $ZnSO_4$, 10% glycerol (final concentration), water and enzyme units as indicated. The incubation was at 48°C for 2 hours and the reaction stopped by the addition of 0.1 ml of 10 mg/ml bovine serum albumin and 1 ml cold 14% perchloric acid. Acid soluble nucleotides were measured by the diphenylamine procedure of Burton (1956). DNA incubated without riboflavin (●–●); S_1 nuclease hydrolysis in dark immediately after addition of riboflavin (○—○); Incubation with riboflavin for 4 hours (△—△); Incubation with riboflavin for 40 hours (▲—▲).


60
40
20
0
% DNA Hydrolyzed
320
640
960
1280
S1 Nuclease (units)

EFFECT OF RIBOFLAVIN AND LIGHT ON THE SECONDARY STRUCTURE OF DNA

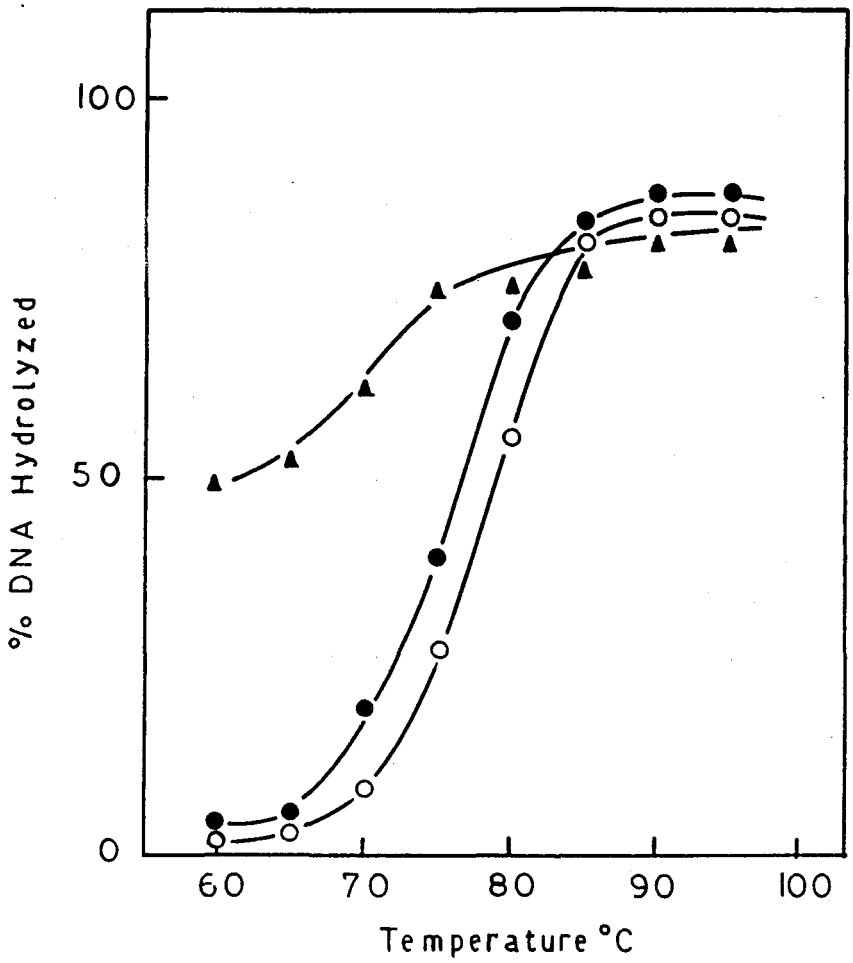
Degradation of riboflavin treated DNA by S_1 nuclease

Figure 21 shows the degradation of native calf thymus DNA, photoilluminated with riboflavin for various time periods, by S_1 nuclease. A higher temperature of 48°C was used for incubation in order to expose the relatively less stable secondary structures in DNA to the enzyme. It is seen that the maximum hydrolysis of DNA preincubated with riboflavin in visible light for 4 hours was greater than that of untreated DNA. Preincubation with riboflavin for 40 hours caused even greater hydrolysis. These observations suggest a destabilization of the secondary structure of DNA as a result of riboflavin treatment. On the other hand, when DNA was hydrolyzed by S_1 nuclease in dark immediately after the addition of riboflavin, the rate of hydrolysis was found to be less than that of native DNA. This would indicate a relative stabilization of the secondary structure of DNA rendering it less susceptible to S_1 nuclease digestion. A similar observation has been made by me with some known DNA intercalating agents such as ethidium bromide and acridine orange (see page 79).

S_1 nuclease has been used in studying the secondary structure of DNA through a variety of approaches; e.g. it has been

Fig. 22 **Thermal denaturation of native and riboflavin treated DNA as measured by the degree of S_1 nuclease digestion.**

DNA was incubated with riboflavin in fluorescent light as described in figure 21. Treated samples containing 300 ug DNA were heated to the desired temperature for 8 minutes and then quickly quenched by the addition of 2 volumes of ice cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 600 units of S_1 nuclease at 40°C for 2 hours. The reaction was terminated and processed as described in figure 21. Native DNA (O-O); Incubation with riboflavin for 4 hours (●—●); incubation with riboflavin for 40 hours (▲—▲).


100
50
0
% DNA Hydrolyzed
60
70
80
90
100
Temperature °C

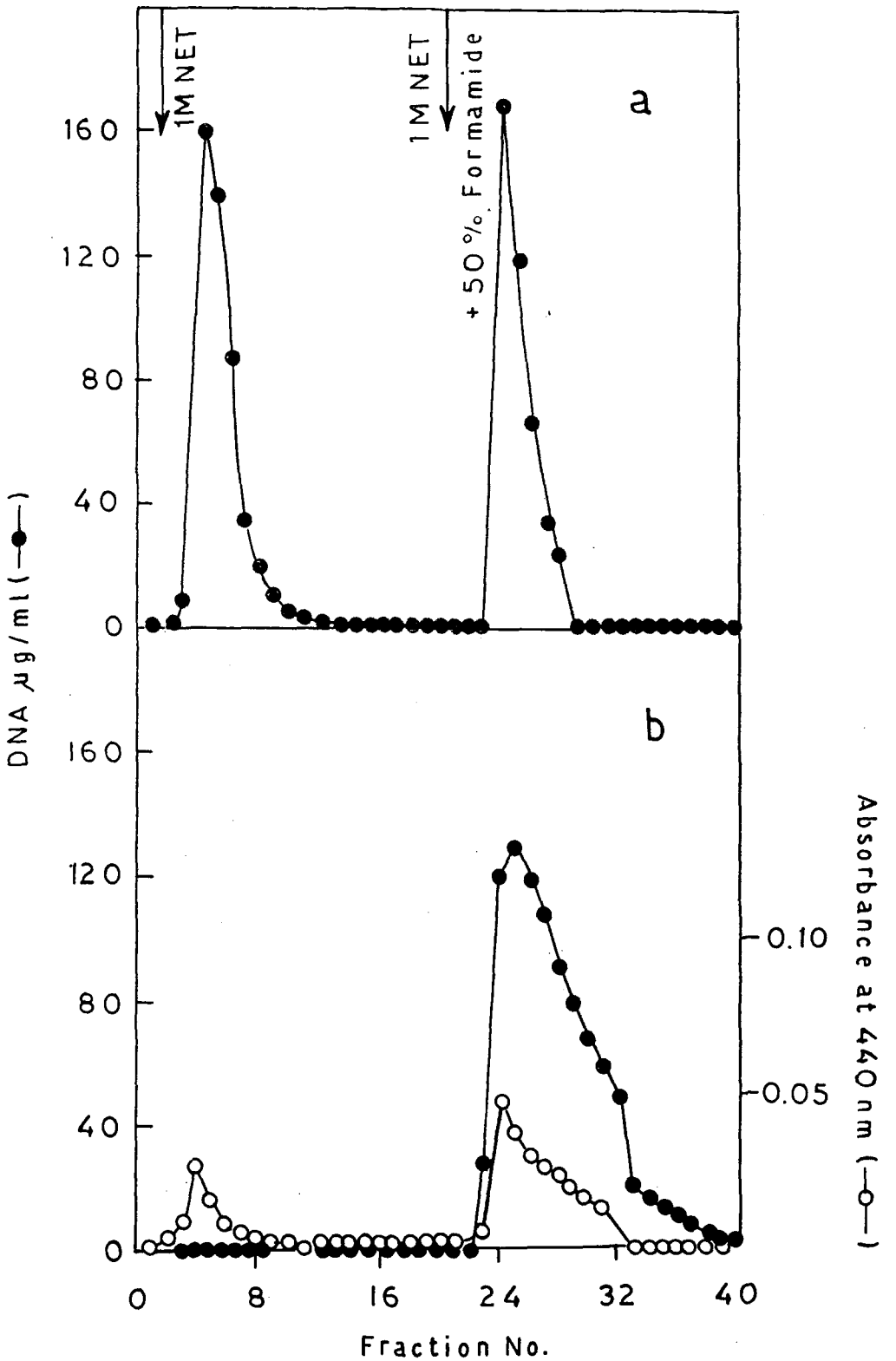
used to determine the thermal melting profile through digestion of the denatured DNA strands. Fig. 22 shows the thermal denaturation of DNA preincubated with riboflavin in light for various times, as measured by the degree of S_1 nuclease digestion. The DNA bp/riboflavin molar ratio used in these experiments was 1:18. After 4 hours of preincubation with riboflavin only a slight decrease in the melting temperature was observed suggesting some disruption of the secondary structure whereas after 40 hours of preincubation no distinct melting temperature could be observed since even at 60°C considerable hydrolysis of DNA by S_1 nuclease was seen. This is indicative of an extensive disruption of the secondary structure resulting possibly by the creation of single strand breaks or regions. However, when the melting temperature was determined immediately after the addition of riboflavin no change from that of control DNA was observed.

BND-cellulose chromatography of DNA treated with riboflavin

BND-cellulose has been used to measure growing points in replicating DNA (Scudiero and Strauss, 1974) and to detect interruptions in human cellular DNA treated with methylmethane sulfonate, an alkylating agent (Karran et al., 1977). Adherence of DNA to BND cellulose, represented by the fraction which elutes with 2.0% caffeine in 1M NET, is considered to be due to two properties: binding of single stranded regions, or binding of

Fig. 23 BND cellulose chromatography of DNA after treatment with riboflavin in fluorescent light.

A (2x4.8 cm) column of BND cellulose was prepared and equilibrated with 0.3M NET. A 3 mg sample of DNA in TNE was incubated with riboflavin (DNA bp/riboflavin molar ratio 1:18) and without riboflavin for 12 hours at 25°C in fluorescent light. The incubated DNA was directly applied to the column and step eluted in 3 ml fractions with 0.3M NET, 1M NET and 1M NET + 50% formamide. DNA was measured by absorption at 260 nm (a) DNA alone; (b) DNA incubated with riboflavin. Recovery in (a) was 90% and in (b) 95%.


1M NET
1M NET
+50% Formamide
a
b
160
120
80
40
0
160
120
80
40
0
0
8
16
24
32
40
Fraction No.
DNA µg/ml (—●—)
Absorbance at 440 nm (—○—)
0.10
0.05

aromatic residues to the resin (Karran et al., 1977).

Figure 23 shows the BND cellulose chromatography of calf thymus DNA photoilluminated in the presence of riboflavin. Control DNA contained approximately equal amounts of completely native DNA (eluting with 1M NET) and DNA with single strand breaks or regions (eluting with 1M NET + 2% caffeine or 50% formamide). On photoillumination almost all the DNA elutes with 1M NET + 50% formamide, apparently due to the creation of single strand breaks in the double stranded molecules. Some 440 nm absorbing material also elutes with 1M NET + 50% formamide which is presumably bound to DNA. The nature of this material has not been investigated further. However, it has been shown that lumiflavin, a photodegradation product of riboflavin, binds to DNA (Kuratomi and Kobayashi, 1977).

Since commercial calf thymus DNA contains single strand breaks as seen above, completely native DNA was prepared by pre-chromatography on BND cellulose of the commercial sample. The 1M NET eluate was dialyzed and used to study the effect of riboflavin in dark and in presence of potassium iodide, a riboflavin triplet excited state quencher. Table VII shows the binding of DNA to BND-cellulose under these conditions. A reduced adherence was seen when the incubation was carried out in dark. When potassium iodide was added and incubation was carried out in light, a further reduction in the fraction of DNA bound to BND-cellulose was observed. It therefore, appears that the adherence to DNA to BND

Table VII

BND-CELLULOSE CHROMATOGRAPHY OF RIBOFLAVIN TREATED DNA

	Reactants	% Adherence to BND-Cellulose
1	DNA alone	11
2	DNA + riboflavin + photoillumination	51
3	DNA + riboflavin (incubated in dark)	41
4	DNA + riboflavin + KI + photoillumination	32

BND cellulose chromatography of riboflavin treated DNA under various conditions. To obtain DNA without single strand breaks or regions, native calf thymus DNA was prechromatographed on BND cellulose. The 1M NET eluate was dialyzed against TNE. DNA was incubated with riboflavin at a DNA bp/riboflavin molar ratio of 1:18 as described in figure 21. The concentration of potassium iodide in experiment 4 was 1mM. 300 ug DNA samples treated as indicated, were applied to the column and elution carried out as described in figure 23 DNA was measured by the diphenylamine method of Burton (1956). Recoveries in all experiments ranged between 90-95 %.

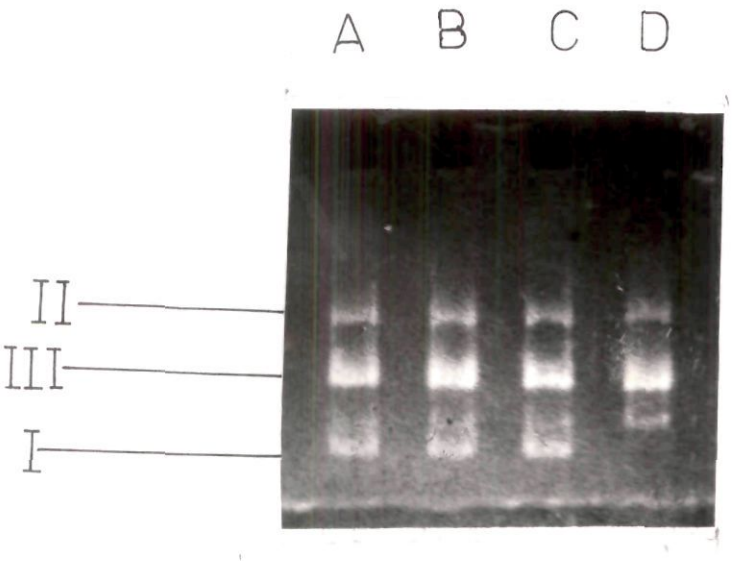
cellulose is presumably due to two factors, creation of single stranded breaks and the possible binding of the aromatic isoalloxazine ring of riboflavin or its degradation products since adherence is also seen on incubation in dark and in the presence of potassium iodide.

Gel electrophoresis of pBR322 DNA treated with riboflavin

I have used plasmid pBR322 DNA and agarose gel electrophoretic technique to detect DNA strand breaks as a result of riboflavin action. Fig. 24 shows the electrophoretic pattern of DNA on treatment with riboflavin under various conditions. Control plasmid DNA, incubated alone showed three bands corresponding to supercoiled, relaxed and linear molecules as indicated. On incubation with riboflavin and illumination, the intensity of bands corresponding to supercoiled molecules and nicked circles undergoes a decrease. In contrast when pBR322 DNA and riboflavin were incubated in dark and in the presence of potassium iodide there was no perceptible change in the intensities of all the three bands. These results indicate that supercoiled DNA molecules and relaxed circles, in the presence of riboflavin and light, are cleaved to form linear molecules. Incubation for longer periods results in the formation of heterogeneous sized molecules resulting in a smear on electrophoresis (results not shown).

Fig. 24 Gel electrophoresis on 1.2% agarose of plasmid pBR322 DNA after illumination with fluorescent light in the presence of riboflavin.

The photoreaction was carried out in TNE containing pBR322 DNA equivalent to 0.25 mM monophosphate nucleotides and 0.05 mM riboflavin. Incubation was done for 12 hours in fluorescent light at 25°C. Bands on the gel correspond to the following conformational forms of plasmid DNA I, supercoiled, closed circular duplex; II, open circular or nicked duplex and III, linear duplex form. Sample (a), DNA illuminated alone; sample (b), incubated with riboflavin in dark; sample (c), incubated with riboflavin and 1mM potassium iodide in light; sample (d) incubated with riboflavin in light.


A
B
C
D
II
III
I

DISCUSSION

It has been reported that riboflavin is the primary chromophore for the action spectrum of visible light mutagenesis in *E. coli*. It is also known that lumiflavin and lumichrome are the photodegradation products of riboflavin and the chemically active semiquinone type free radical is formed by the reduction of lumiflavin with visible light. A mechanism of interaction involving hydrogen bonding between lumiflavin and guanine moieties of DNA has been proposed if intercalation of the flavin between guanine and another base of the DNA strand could occur (Kuratomi and Kobayashi, 1977). Some support for the intercalation model is provided by the finding that the formation of superoxide anion is considerably faster in presence of DNA than RNA (Korycka-Dahl and Richardson, 1980). In our results significant binding of DNA to BND cellulose occurs (Table VII) even on incubation with riboflavin in dark and in the presence of potassium iodide, the riboflavin triplet excited state quencher. Triplet state quenchers shorten the life time of triplet riboflavin and prevent it from undergoing photoreduction (Korycka-Dahl and Richardson, 1980). Under these conditions no breaks are introduced in the supercoiled molecules as shown in Figure 24. This would indicate that the binding of DNA to BND cellulose in dark is not due to the formation of single strand breaks and must be accounted for by the binding of aromatic residues to the

resin. possibly this represents the intercalated complex of riboflavin with DNA. Kuratomi and Kobayashi (1977) have presented evidence for the binding of lumiflavin to DNA possibly through the interaction of the flavin with guanine bases of DNA. Speck et al. (1976) concluded that the increase in buoyant density of DNA samples illuminated in the presence of riboflavin is primarily due to a chemical alteration of DNA (perhaps a DNA base) and not due to generation of single stranded breaks. In contrast Korycka-Dahl and Richardson (1980) have shown that riboflavin sensitized photoreaction causes single and double strand breaks in supercoiled PM2 DNA. Our results therefore,are in support of the latter observation. In addition, the binding of DNA to BND cellulose, treated with riboflavin in dark and in the presence of triplet excited state quencher, potassium iodide, indicates that riboflavin itself interacts with DNA possibly through its flavin moiety.

INTERACTION OF QUERCETIN WITH DNA

Action of S_1 nuclease on quercetin treated DNA:

Most of the flavonoids that have been found mutagenic require metabolic activation except quercetin; the mutagenicity of quercetin is however enhanced by rat liver enzymes(Nagao et al., 1978).

It was therefore considered of interest to determine whether quercetin binds to DNA *in vitro*. With this object in view the following series of experiments were carried out. On binding to DNA quercetin may create a distortion in the secondary structure which may be less stable and therefore detectable by S_1 nuclease. Conversely, it may intercalate between base pairs causing an increase in the melting temperature. On binding to DNA it may also protrude outside the double helix which can be determined by BND cellulose chromatography (Hurley and Petrusek, 1979).

Figure 25 compares the rate of hydrolysis of native calf thymus DNA by S_1 nuclease in the absence and presence of quercetin at a DNA bp/quercetin molar ratio of 2.5:1 after various periods of treatment. A higher temperature of 48°C was used for enzyme incubation in order to expose the relatively less stable secondary structures in DNA to the enzyme. In Fig. 25a the enzyme was added to the DNA immediately after its preincubation with the drug. Removal of the drug by dialysis would have increased its period of interaction with DNA. Therefore using denatured DNA it was earlier shown that S_1 nuclease is not inhibited at the concentration of quercetin tested (Table VIII). It is seen that the rate of DNA hydrolysis in presence of quercetin is only about 50% of that in its absence. However after 10 hours of preincubation of DNA with the drug and dialysis, this difference is considerably reduced (Fig. 25b). Indeed, after

Fig. 25 Effect of S_1 nuclease on DNA pretreated with quercetin for various time periods.

3 mg native calf thymus DNA in 1.5 ml TNE was preincubated at 30°C with 540 ug of quercetin to obtain a DNA bp/quercetin molar ratio of 2.5:1 for various time periods. Native DNA alone was also incubated for the same period under similar conditions. After incubation both native and quercetin treated DNAs were dialyzed against 1.0 litre of TNE overnight at room temperature. S_1 nuclease reaction was started by the addition of S_1 nuclease standard reaction buffer, glycerol to a final concentration of 10% and 40000 units of S_1 nuclease and the volume made up to 5 ml. The incubation was carried out at 48°C and 1 ml aliquots (containing 500 ug substrate DNA) were drawn at the time intervals indicated. The reaction was terminated and processed as described earlier.

PANEL (a) Incubation with quercetin for 1 hr

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)

PANEL (b) Incubation with quercetin for 10 hrs

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)

PANEL (c) Incubation with quercetin for 24 hrs

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)

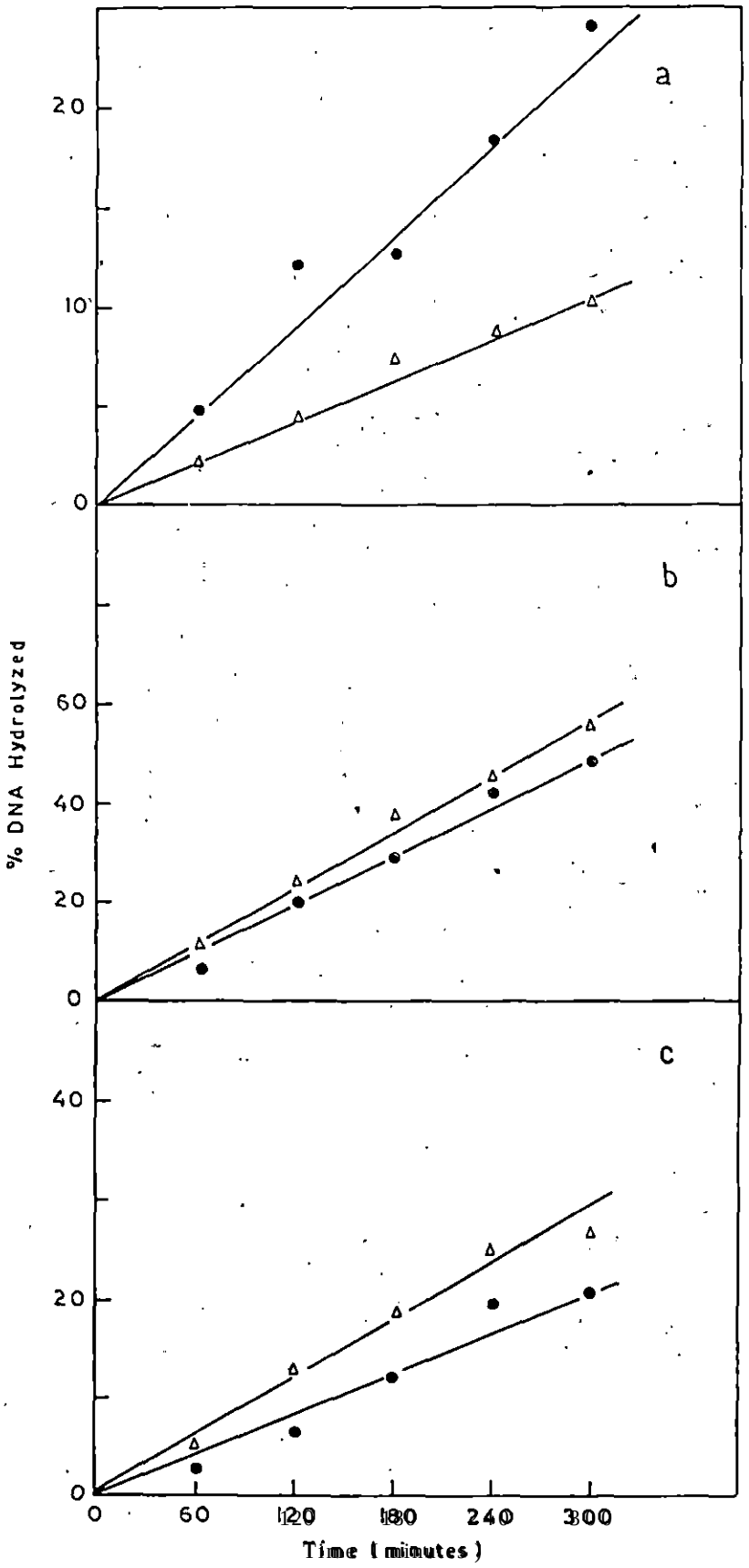

a
b
c
20
10
0
60
40
20
0
40
20
0
% DNA Hydrolyzed
0
60
120
180
240
300
Time (minutes)

Fig. 26 Thermal denaturation of native and quercetin treated DNA as measured by the degree of S_1 nuclease digestion.

3 mg DNA in 1.5 ml TNE was preincubated at 30°C with 540 ug of quercetin (in TNE) to obtain a DNA bp/quercetin molar ratio of 2.5:1 at 37°C for various time periods as described in figure 25. Treated samples containing 300 ug substrate DNA were heated to the desired temperature for 8 minutes and then quickly quenched by the addition of 2 volumes of ice cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 800 units of S_1 nuclease at 40°C for 3 hours. The reaction was terminated and processed as described earlier.

PANEL (a) Incubation with quercetin for 1 hour

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)

PANEL (b) Incubation with quercetin for 10 hours

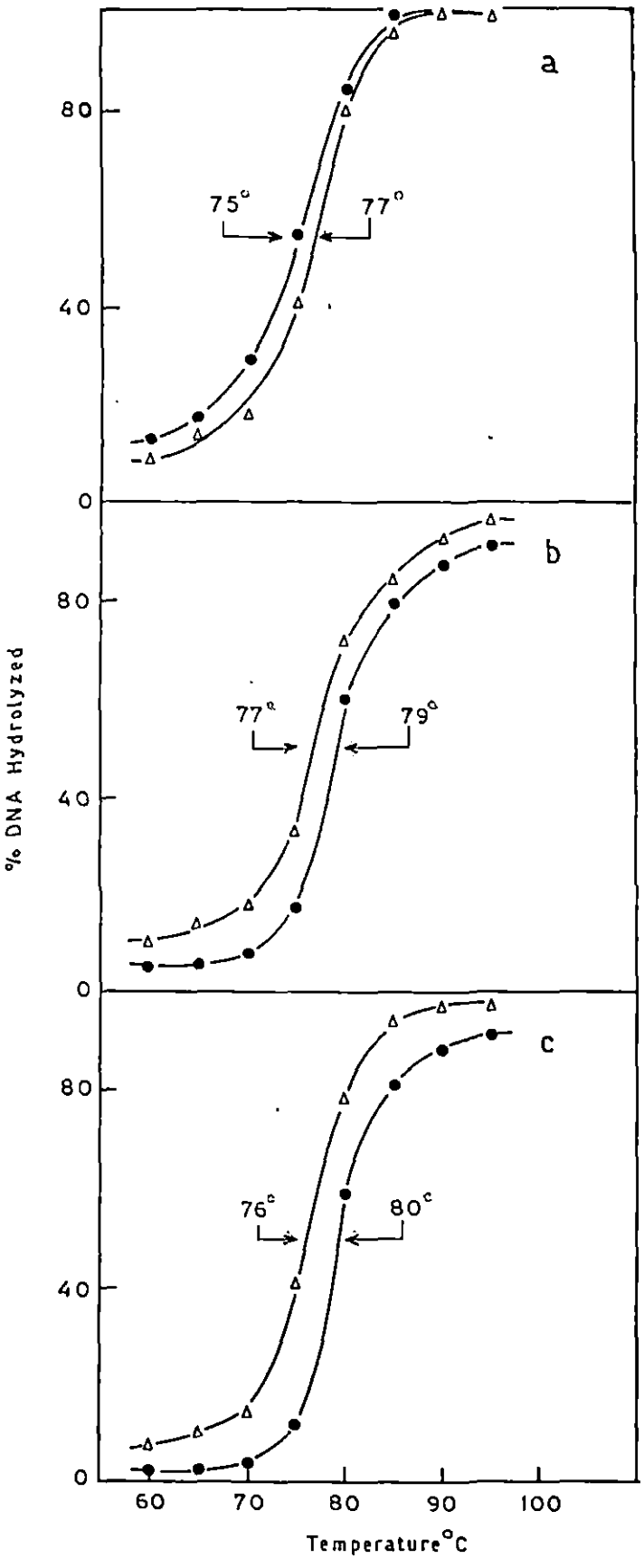
Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)

PANEL (c) Incubation with quercetin for 24 hours

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)


a
b
c
75°
77°
77°
79°
76°
80°
% DNA Hydrolyzed
Temperature°C
0
40
80
60
70
80
90
100

24 hours of preincubation the rate of hydrolysis of treated DNA is found to be greater than the control sample (Fig. 25c). Ligands that bind to DNA by intercalation enhance the melting temperature of the duplex molecule. This would be expected to decrease the rate of hydrolysis of duplex DNA by S_1 nuclease at 48°C. On the other hand production of single strand nicks or regions in duplex would enhance the rate of hydrolysis under these conditions.

When S_1 nuclease was used to determine the thermal melting profile of DNA in presence of quercetin (Fig. 26a) no significant difference in the thermal melting profile from that of native DNA was observed. A slight increase in melting temperature in the presence of quercetin (77°C versus 75°C) was however seen. At 75°C and below, the S_1 nuclease susceptible hydrolysis was consistently found to be greater in the absence of quercetin. Native DNA was therefore heated at 75°C in the presence and absence of quercetin for various times before quenching in ice and hydrolysis with S_1 nuclease. The results are shown in Fig. 27(a). It is seen that upto at least 2 minutes the strand separation is greater in control DNA samples than in the presence of quercetin. This would suggest the possibility of quercetin binding to DNA through noncovalent interactions. Figure 27(b) shows the same experiment with DNA preincubated for 24 hours with quercetin. Here the strand separation is consistently found to be greater in the drug treated sample indicating an altered

Fig. 27 Effect of heating quercetin treated DNA at 75°C on its degradation by S_1 nuclease.

25 mg of native calf thymus DNA in 12.5 ml TNE was preincubated at 37°C with 4.5 mg of quercetin (in TNE) to achieve a DNA bp/quercetin molar ratio of 2.5:1 for 1 hour and 24 hours respectively. Native DNA alone without quercetin treated in a similar way served as control. After incubation each sample was dialyzed against 1 litre of TNE overnight at room temperature. Treated samples containing 300 ug DNA in 0.3 ml TNE were heated at 75°C for the indicated time periods and then quickly quenched by the addition of 2 volumes of ice cold S_1 nuclease standard reaction buffer. This was followed by addition of 300 units of S_1 nuclease and incubation at 40°C for 3 hours. The reaction was terminated and processed as described earlier.

PANEL (a) Preincubation for 1 hour

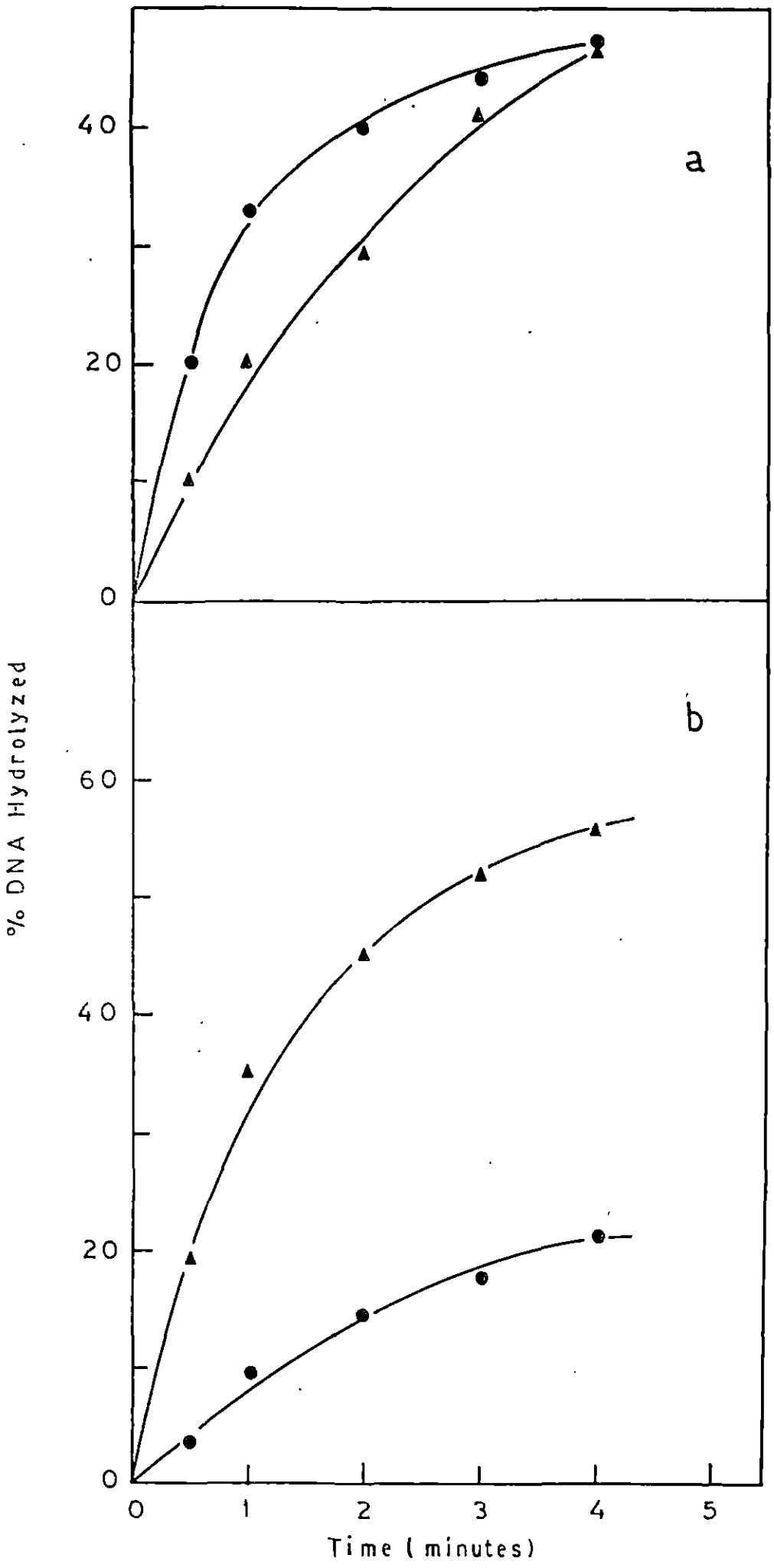
Native DNA (●——●)

Quercetin treated DNA (▲——▲)

PANEL (b) Preincubation for 24 hours

Native DNA (●——●)

Quercetin treated DNA (▲——▲)


a
b
40
20
0
60
40
20
0
0
1
2
3
4
5
% DNA Hydrolyzed
Time (minutes)

secondary structure of DNA on prolonged incubation with the drug. The thermal melting profile was also determined using DNA preincubated with quercetin for 10 hours and 24 hours along with control sample treated similarly but without quercetin. The melting temperature of drug treated samples was found to be lesser by 2°C and 4°C respectively. These observations suggest a destabilization of the secondary structure of DNA on extended treatment with quercetin.

The metabolically activated form of the potent carcinogen benzo (a) pyrene binds to DNA covalently at guanine residues. The complexes have been reported to be susceptible to digestion with S_1 nuclease suggesting that the DNA at the carcinogen binding site is in effect single stranded (Pulkrabeck et al., 1977). Native DNA was digested with S_1 nuclease after preincubation with quercetin for 1 hour and 24 hours and chromatographed on Sepharose 6B. Figure 28 shows the elution profiles along with denatured and native DNA treated similarly. The top panel indicates that heat denatured DNA is almost completely digested and yields small oligonucleotides which elute at the peak position of 51 ml. Figure 28(b) shows that a small amount of native DNA is digested under the same conditions. Presumably, this reflects digestion of the ends of the DNA fragments. The bottom panel shows the elution profile after incubation in presence of quercetin for 1 hour. However, when 24 hours drug treated sample was digested with S_1 nuclease, various sized

Fig. 28 Sepharose 6B chromatography of native and quercetin treated DNA degraded with S_1 nuclease.

The column (2x22 cm) was thoroughly equilibrated with TE buffer (0.2M NaCl, 0.01M Tris HCl pH 7.5 and 10^{-3}M EDTA). Denatured DNA was prepared as described earlier. Quercetin treated DNA was prepared by treating separately 3 mg DNA samples in 1.5 ml TNE with adequate amount of quercetin to achieve a DNA bp/quercetin molar ratio of 2.5:1 at 37°C for 1 hour and 10 hours respectively. This was dialysed overnight against 1 litre of TNE at room temperature. The reaction mixture in a total volume of 2.8 ml contained 3 mg DNA (denatured, native or quercetin treated) S_1 nuclease reaction buffer and 3200 units of S_1 nuclease. Incubation was carried out at 37°C for 1 hour. The reaction was terminated by adding 0.2 ml of 0.1M EDTA followed by immediate loading on the column. Elution was performed with TE buffer and 3 ml fractions were collected at the rate of 12-15 ml/hour. DNA was estimated in 1 ml of the fraction by the diphenylamine procedure (Schneider, 1957).

PANEL (a) Denatured DNA treated with S_1 nuclease.

PANEL (b) Native DNA treated with S_1 nuclease.

PANEL (c) Native DNA pretreated with quercetin at 37°C for 1 hour prior to incubation with S_1 nuclease.

PANEL (d) Native DNA pretreated with quercetin at 37°C for 10 hours prior to incubation with S_1 nuclease.

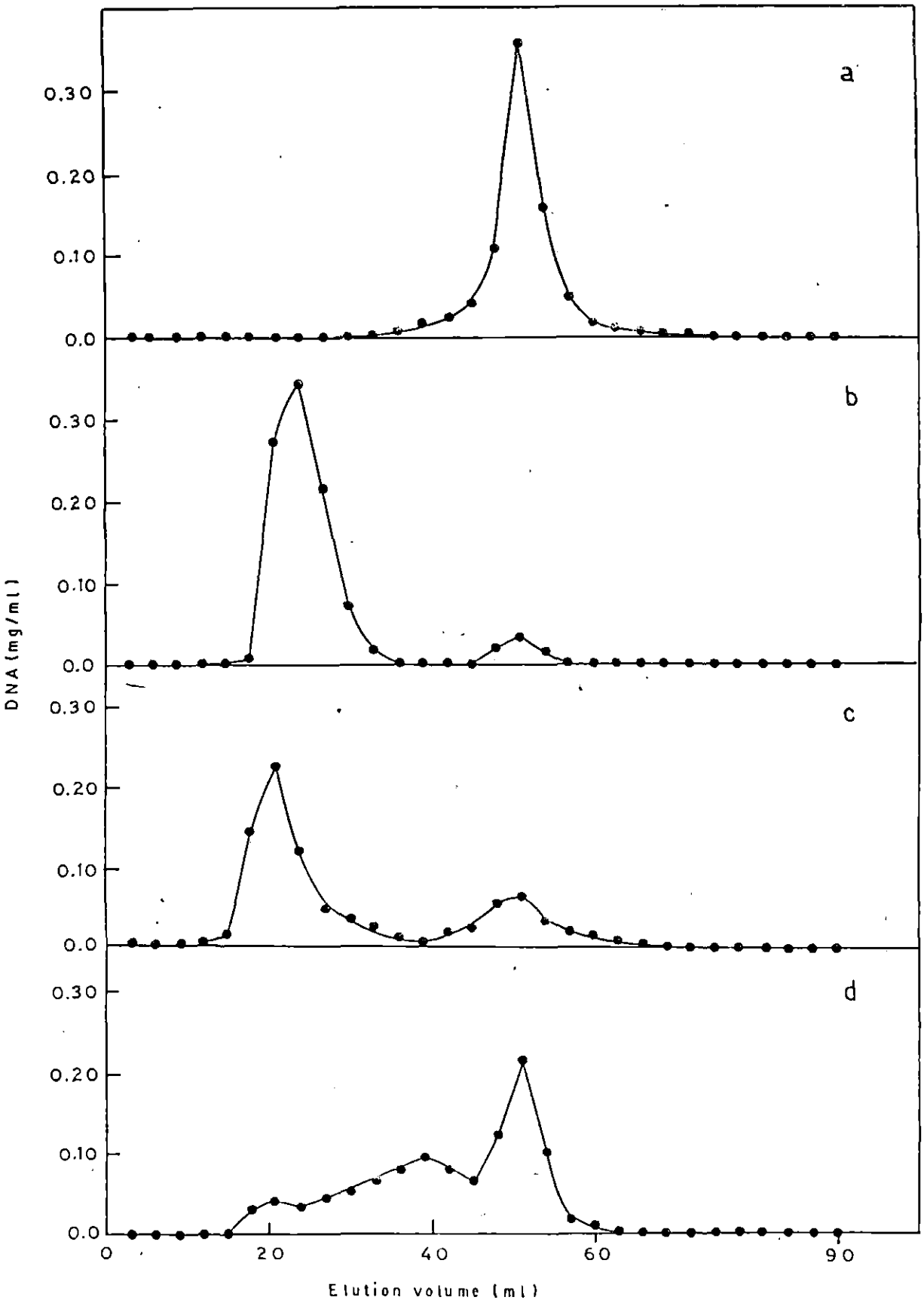
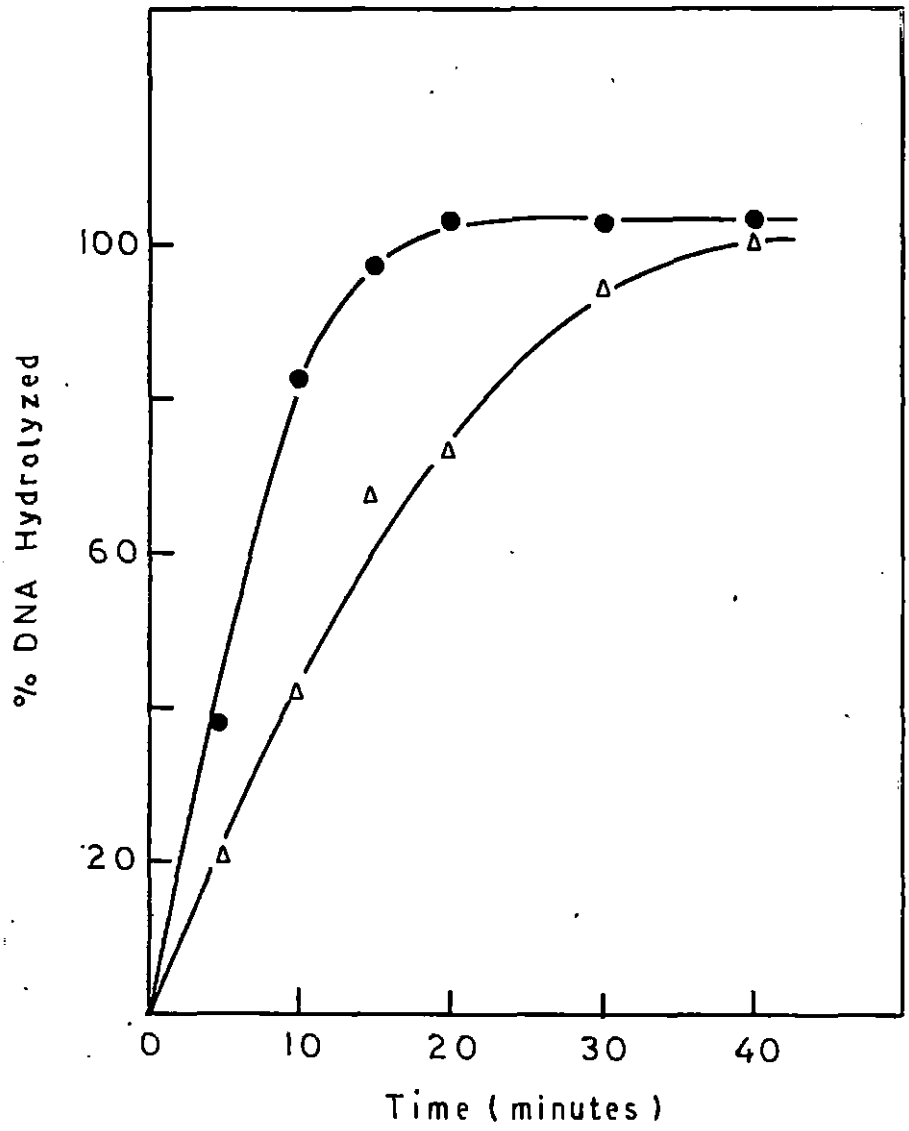

a
b
c
d
0.30
0.20
0.10
0.0
DNA (mg/ml)
0
20
40
60
90
Elution volume (ml)

Fig.29: Enzymatic hydrolysis of native and quercetin treated DNA by a mixture of S_1 nuclease and DNase I.

3 mg native calf thymus DNA in 1.5 ml TNE was preincubated at 37°C for 1 hour with adequate amount of quercetin to achieve a DNA bp/quercetin molar ratio of 2.5:1. After preincubation, acetate buffer (0.1M final concentration), $ZnSO_4$ and $MgCl_2$ to a final concentration of 1mM and a mixture containing DNase I (20 ug) and S_1 nuclease (3200 units) were added in a total volume of 3.0 ml. The incubation was carried out at 37°C. 0.4 ml aliquots containing 400 ug substrate were removed at the time intervals indicated and added to 0.2 ml of 2 mg/ml BSA and 1 ml cold 14 % PCA. Further processing for the determination of acid soluble nucleotides was the same as described earlier.

Native DNA (●——●)

Quercetin treated DNA (Δ——Δ)


% DNA Hydrolyzed
100
60
20
0
10
20
30
40
Time (minutes)

degraded molecules were produced, a major part of which was equivalent in size to the digestion products of heat denatured DNA. These results are in agreement with those of Figure 25 and 26 and suggest that initially quercetin may bind to DNA in a manner which stabilizes its secondary structure but prolonged treatment leads to the disruption of the double helix.

The experiment shown in Figure 29 measures the rate at which a mixture of DNase I and S_1 nuclease digested the quercetin treated and untreated DNA. It was earlier shown that DNase I is not inhibited at the concentration of quercetin tested (Table VIII). DNA was pretreated with quercetin for 1 hour at a DNA bp/drug molar ratio of 2.5:1 before addition of enzyme mixture. After 10 minutes of enzyme treatment only 40% of quercetin treated DNA is hydrolyzed whereas the hydrolysis of untreated DNA is more than 80%. This observation again supports the idea that the initial interaction of quercetin has a stabilizing effect on the double helix.

Gel electrophoresis of phage lamda DNA treated with quercetin in light

The above results clearly indicate that treatment of native DNA with quercetin leads to the formation of single stranded breaks or regions which are susceptible to the action of S_1 nuclease. Flavonoids are known to undergo various oxidative degradations in the presence of light and oxygen. Under these

Table VIII

ACTIVITY OF S_1 NUCLEASE AND DNase I IN PRESENCE OF VARYING CONCENTRATIONS OF QUERCETIN

DNA bp/quercetin molar ratio	% DNA hydrolyzed	
	S_1 Nuclease	DNase I
CONTROL (no drug)	100.00	100.00
20:1	97.81	96.11
10:1	94.93	92.13
5:1	93.90	86.51
2.5:1	92.40	84.27

For S_1 nuclease assay the reaction mixture contained 400 ug denatured DNA in 0.2 ml TNE, sufficient amount of quercetin to obtain the desired DNA bp/quercetin molar ratios, stock S_1 nuclease reaction buffer, 480 units of S_1 nuclease and water to make up the volume to 1.0 ml. The incubation was at 40°C for 2 hours. The reaction was terminated and processed as described earlier. For DNase I assay the reaction mixture in a total of 1 ml contained 400 ug native calf thymus DNA (in 0.2 ml TNE) adequate amount of quercetin to achieve the desired DNA bp/ quercetin molar ratios, Tris HCl pH 7.2 and $MgCl_2$ (final concentration 0.1M and 1mM respectively) and 4 ug of DNase I. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated and processed as described earlier.

conditions quercetin has been shown to give rise to a mixture of a depside and two carboxylic acids in addition to CO and CO_2 (Matsuura and Matsushima, 1967 and Matsuura et al., 1970). It is possible that one of these degradation products may react with the DNA bases or phosphates giving rise to single or double stranded breaks. Riboflavin is considered to react with guanine moieties in DNA in the presence of visible light and oxygen (Kuratomi and Kobayashi, 1977 and page 98 of this thesis). In order to test the above possibility the experiments shown in figures 30 and 31 were carried out. Phage lambda DNA was incubated with quercetin at 37°C for various time periods and electrophoresed on agarose gel. Control untreated DNA showed a sharp band whereas incubation with quercetin for 1 hour or more in the presence of 1640 lux of fluorescent light gives rise to a smear suggesting the generation of smaller heterogeneous sized molecules. The photo-oxidation of nucleic acid bases may involve superoxide anion which may undergo secondary reaction to form hydrogen peroxide, singlet oxygen or an hydroxy radical. These "active oxygen species" being strong oxidizing agents may be involved in the oxidation of nucleic acids. The other possibility can be that triplet excited state quercetin, may react with DNA and leads to its photodegradation. Figure 31 shows that DNA when incubated with quercetin in dark, does not get degraded. When the reaction was carried out in presence of triplet excited state quencher, potassium iodide, no protection of

Fig. 30 Gel electrophoresis of bacteriophage lambda DNA after illumination with fluorescent light in the presence of quercetin for various time periods.

The photoreaction was carried out in 5ul TNE containing 1 ug phage lambda DNA and adequate amount of quercetin to achieve a DNA bp/quercetin molar ratio of 2.5:1. Incubation was done in fluorescent light at 37°C for various time periods. The tubes were heated at 70°C for 5 minutes prior to quenching in ice. Sufficient water was added to make up the volume to 15 ul. 5 ul of the sample buffer was added and half the volume was loaded on a 1.2 % agarose horizontal slab gel. The gel was electrophoresed submerged under the electrophoresis buffer (TBE) which contained 1 ug/ml ethidium bromide and photographed as described. Samples A, B, C, D, E, and F indicate DNA photoilluminated for 0 hr, 0.5 hr, 1 hr, 2 hrs, 4 hrs and 8 hrs respectively.

A B C D E F

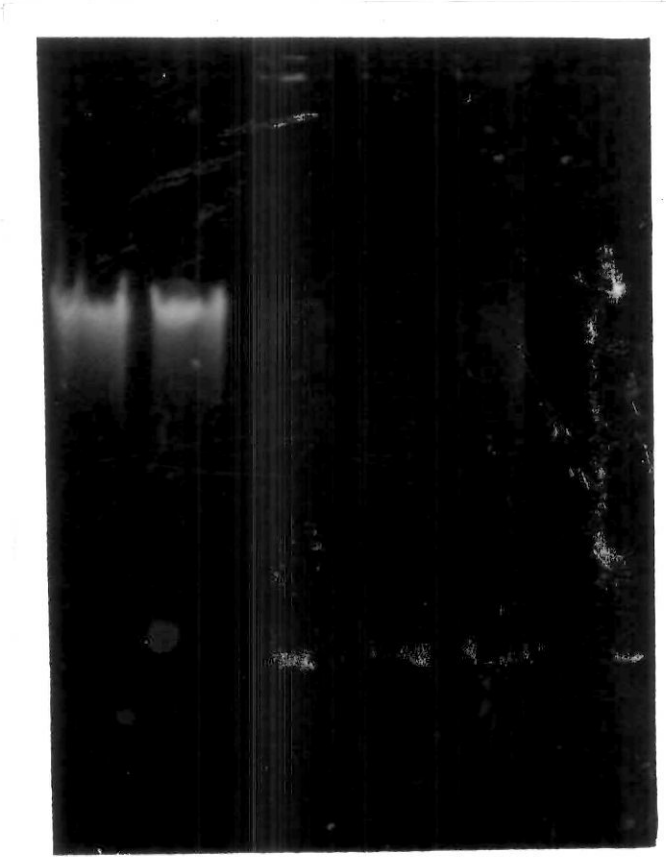
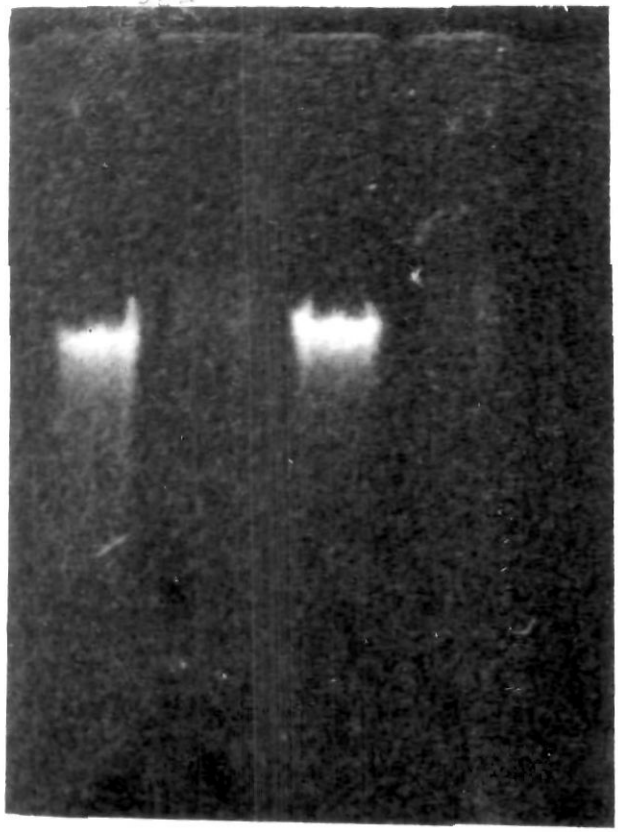


Fig. 31 Gel electrophoresis of phage lambda DNA photoilluminated with quercetin and with potassium iodide.

The photoreaction mixture was the same as given in figure 30 except that the sample D had KI to a final concentration of 1mM. Incubation was done in fluorescent light for 2 hours at 37°C sample (A); DNA illuminated alone; (B), incubated with quercetin in light; (C), incubated with quercetin in dark; and (D), incubated with quercetin and 1mM potassium iodide.

A B C D



DNA was seen. Thus the mechanism of photodegradation of DNA in the presence of quercetin remains obscure and needs to be explored further. Use can be made of various active oxygen scavengers such as superoxide dismutase, which destroys superoxide anion, catalase which destroys hydrogen peroxide and B -carotene which is an efficient singlet oxygen quencher.

SUMMARY AND CONCLUSION

These studies were aimed at investigating the changes in secondary structure of DNA due to the binding of various mutagenic drugs. Some representative intercalating and non intercalating dyes and drugs were chosen. In addition, the effect of the ubiquitous vitamin riboflavin and mutagenic flavonoid quercetin on DNA was also studied. Certain techniques and tools such as hydroxyapatite chromatography, BND-cellulose chromatography and single strand specific S_1 nuclease were used. The usefulness of these methods in nucleic acid studies is already well documented. Our studies are therefore, an extension of these methods in elucidating the structure of various DNA-drug complexes.

The binding properties of DNA complexed with intercalating agents (ethidium bromide, acriflavine, actinomycin D and acridine orange) to hydroxyapatite (HA) were found to be quite different than of native DNA alone. DNA was eluted from HA columns complexed with the intercalating agent and at a significantly lower phosphate molarity than untreated DNA. With non-intercalating agents (spermine and berenil) no such alteration in the elution pattern was observed and DNA and the ligand eluted separately. When ethidium bromide was extracted from chromatographed DNA and the DNA rechromatographed on hydroxyapatite, it again behaved as double stranded material. The degree of binding of ethidium bromide to DNA was

quantitated on HA and was found to be comparable to the Scatchard plot analysis of the spectrophotometric data of the binding of the dye to DNA. These investigations provide an additional method which is simple and fairly rapid of determining the mode of binding to DNA of polycyclic aromatic and other ligands.

During the course of these studies a new and sensitive method for the colorimetric estimation of the trypanocidal drug, berenil was also developed and its binding in vitro to hemoglobin and other serum fractions established.

S_1 nuclease hydrolysis and benzoylated naphthoylated DEAE cellulose (BND cellulose) chromatography was used to demonstrate that alkylation of DNA by dimethyl sulfate at neutral pH leads to the production of partially denatured molecules under conditions where no significant depurination occurs. DNA was alkylated with increasing concentrations of the alkylating agent and subjected to enzymatic degradation and binding to BND cellulose. An increasing degree of DNA hydrolysis and adherence to BND cellulose was seen. On hydroxyapatite chromatography the alkylated DNA still eluted at the position of double stranded molecules suggesting the presence of partially denatured regions. The presence of salt had a preventive effect on such denaturation.

S_1 nuclease was also used to explore the changes in the secondary structure of DNA complexed with intercalating and

non intercalating agents. A progressive decrease in the rate and extent of hydrolysis of DNA complexed with increasing relative concentrations of the known intercalator, ethidium bromide was observed. With increasing enzyme concentration, the decrease in maximum DNA hydrolyzed was considerably greater in the case of ethidium bromide than with spermine, a non intercalating agent. S_1 nuclease was also used to determine the thermal melting profile of native DNA complexed with increasing molar ratios of ethidium bromide and acridine orange. The melting profile was found to be biphasic with both the drugs. Also, complete hydrolysis of DNA was not observed even at 95°C and progressively decreasing maximum hydrolysis was seen. These observations are in agreement with the property of intercalating agents of stabilizing the secondary structure of DNA. However, such biphasic melting profiles were characteristically absent in case of DNA treated with non intercalating agents like streptomycin sulfate and spermine. These results are possibly accounted for, by the property of intercalating agents of stabilizing the secondary structure of DNA and their reported preference in binding to G-C base pairs.

Dimethylsulfoxide and formamide are two solvents which destabilize the secondary structures in nucleic acids. The usefulness of S_1 nuclease can be enhanced if it can be shown to be active in these solvents. S_1 nuclease exhibited no inhibition of activity with alkylated DNA in presence of 5%

dimethyl sulfoxide and formamide. Presence of increasing concentration of sodium chloride affected the hydrolysis of denatured, alkylated and depurinated DNA by S_1 nuclease to about the same degree. Treatment of depurinated DNAs prepared at 40°C, 50°C, 60°C and 70°C at pH 3.5 with S_1 nuclease exhibited an increasing degree of maximum hydrolysis which was consistent with the fact that higher temperature leads to increased depurination causing increasing separation of the two strands.

S_1 nuclease hydrolysis and BND cellulose chromatography were employed to study the effect of riboflavin and visible light on DNA. Native calf thymus DNA was incubated with riboflavin in the presence of fluorescent light for various time periods (4 hours and 40 hours respectively) and subjected to S_1 nuclease hydrolysis. An increasing degree of DNA degradation was seen suggesting a destabilization of the secondary structure. A decrease in melting temperature was also observed. Incubation with riboflavin and illumination caused adherence to BND cellulose indicating the production of single stranded regions or breaks in the native double stranded molecules. However, when incubation was done in dark and in the presence of triplet excited state quencher, potassium iodide, a reduced adherence of DNA to BND cellulose was seen. Plasmid pBR322 DNA was also treated with riboflavin under these conditions and subjected to agarose gel electrophoresis. No degradation could be seen in dark incubated and potassium iodide

treated sample. These results indicate that the adherence of DNA to BND cellulose in dark is possibly due to the binding of aromatic residues to the resin suggesting the formation of a complex between riboflavin and DNA.

In vitro experiments were also conducted to study the DNA modifying effects of the mutagenic flavonoid quercetin. The rate of DNA hydrolyzed after 4 hours of pretreatment with quercetin was found to be only about 50% of that in its absence. However, after 10 and 24 hours of preincubation with the drug, the rate of S_1 nuclease hydrolysis of treated DNA was found to be greater than that of native DNA. Thermal melting profiles of DNA preincubated for 10 and 24 hours respectively indicate a slight decrease in melting temperatures ($2^{\circ}C$ and $4^{\circ}C$ respectively). These results suggested a destabilisation of the secondary structure either through the creation of partial single stranded regions or single stranded breaks in DNA. Sepharose 6B chromatography of native DNA which had been digested with S_1 nuclease after preincubation with quercetin for 24 hours indicate the production of various sized degraded molecules a major part of which was equivalent in size to the digestion products of heat denatured DNA. Digestion of quercetin treated and untreated DNA by a mixture of DNaseI and S_1 nuclease showed that after 10 minutes of enzyme treatment only 40% of quercetin treated DNA is hydrolyzed whereas the hydrolysis of untreated DNA is more than 80%.

These results indicate that the initial interaction of quercetin with DNA may have a stabilizing effect on its secondary structure but prolonged treatment leads to an extensive disruption of the double helix.

Abelson, J. and Thomas, C.A. (1966) J. Mol. Biol. 18, 262.

Ambrose, A.M., Robbins, D.J. and De Eds, F. (1952) J. Am. Pharm. Assoc. 41, 119.

Ando, T. (1966) Biochim. Biophys. Acta 114, 158.

Bauer, W. and Vinograd, J. (1968) J. Mol. Biol. 33, 144.

Beard, P., Morrow, J.F. and Berg, P. (1973) J. Virol. 12, 1303.

Behrman, R.E., Brown, A.K., Currie, M.R., Hastings, J.W., Odell, G.B., Schaffer, R,Setlow, R.B., Vogl, T.P., Wurtaman, F.J., Anderson, R.K., Kostkowski, H.J. and Simopoulos, A.P., (1974) J. Pediat. 84, 135.

Berk, A.J., and Sharp, F.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1274.

Bernardi, G. (1962) Biochem. J. 83, 62.

Bernardi, G. (1965) Nature (London), 206, 779.

Bernardi, G., Carnevali, F., Nicolaieff, A., Piperno, G. and Tecce, G. (1968) J. Mol. Biol. 37, 493.

Bernardi, G. (1969) Biochim. Biophys. Acta. 174, 449.

Bernardi, G., Faures, M., Piperno, G. and Slonimski, P.L. (1970) J. Mol. Biol. 48, 23.

Bernardi, G. (1971) in Methods Enzymol. (Colowick, S.P. and Kaplan, N.O. eds.), vol. 25 p. 95, Academic Press Inc., New York.

Bittman,R. (1969) J. Mol. Biol. 46, 252.

Bjeldanes, L.F. and Chang, G.W. (1977) Science 197, 577.

Bourgaux, R.D., Van Teighem, M. and Bourgaux, P. (1967) J. Gen. Virol. 1, 580.

Bradley, M.O. and Sharkey, N.A. (1977) Nature 266, 724.

Brenner, S., Barnett, L., Crick, F.H.C. and Orgel, A. (1961) J. Mol. Biol. 3, 121.

Brown, J.P. and Doetrich, P.S. (1979) Mutat. Res. 66, 223.

Brown, J.P. (1980) Mutat. Res. 75, 243.

Burnotte, J. and Verly, W.G. (1972) Biochim. Biophys. Acta 262, 449.

Burton, K. (1956) Biochem. J. 62, 315.

Case, S.T. and Baker, R.F. (1975) Anal. Biochem. 64, 477.

Case, S.T., Mongeon, R.L. and Baker, R.F. (1974) Biochim. Biophys. Acta 349, 1.

Cohen, G. and Eisenburg, H. (1969) Biopolymers 8, 45.

Colman, A., Byers, M.J., Primrose, S.B. and Lyons, A. (1978) Eur. J. Biochem. 91, 303.

Crawford, L.V. and Waring, M.J. (1967) J. Mol. Biol. 25, 23.

Crosa, J.H., Brenner, D.J. and Falkow, S. (1973) J. Bacteriol. 115, 904.

Dodgson, J.B. and Wells, R.D. (1977) Biochemistry 16, 2374.

Duncan, J., Hamilton, L. and Friedberg, E. (1976) J. Virol. 19, 338.

Fiel, R.J., Howard, J.C. Mark, E.H. and Dattagupta, N. (1979) Nucleic Acids Res. 6(9) 3093.

Fuller, W. and Waring, M.J. (1964) Ber. Bungsenges. Phys. Chem. 68, 805.

Germond, J.R. Vogt, V.M. and Hirl, B. (1974) Eur. J. Biochem. 43, 591.

Gillam, S., Milward, S., Bleu, D., Von Yigestrom, Wimmer, E. and Tenner, G.M. (1967) Biochemistry 6, 3043.

Godson, G.N. (1973) Biochim. Biophys. Acta, 303, 59.

Godson, G.N. (1974) Virology 58, 272.

Hadi, S.M. and Goldthwait, D.A. (1973) Biochemistry 10, 4986.

Hermann, K. (1976) J. Food Technol. 11, 433.

Hirono, I., Ueno, I, Hosaka, S. Takanashi, M., Matsushima, T., Sugimura, T., and Natori, S. (1981) Cancer Lett. 13, 15.

Hjerten, S. (1959) Biochim. Biophys. Acta 31, 216.

Hogan, M.E., Dattagupta, N. and Whitlock Jr., J.P. (1981) J. Biol. Chem. 256 (9) 4504.

Holloman, W.K., Godson, G.N., Moessli, R. and Radding, C.M. (1975) Proc. Natl. Acad. Sci. USA 72, 2394.

Hsiung, J.H., Lown, W. and Johnson, D. (1976) Can. J. Biochem. 54, 1047.

Hurley, L.H. and Petrusek, R. (1979) Nature 289, 529.

Hutton, J.R. and Wetmur, J.G. (1975) Biochem. Biophys. Res. Commun. 66, 942.

Iyer, V.N. and Rupp, W.D. (1971) Biochim. Biophys. Acta 228, 117.

Karran, P., Higgins, N.P. and Strauss, B. (1977) Biochemistry 16, 4483.

Kastrup, R.V., Young, M.A. and Krugh, T.R. (1978) Biochemistry 17, 4855.

Kasumatso, H. and Vinograd, J. (1974) Ann. Rev. Biochem. 43, 695.

Kersten, W., Kersten, M. and Szybalski, W. (1966) Biochemistry 5, 236.

Korycka-Dahl, M. and Richardson, T. (1978) J. Dairy Sci. 61, 400.

Korycka-Dahl, M. and Richardson, T. (1980) Biochim. Biophys. Acta. 610, 229.

Kuratomi, K. and Kobayashi, Y. (1977) Biochim. Biophys. Acta. 476, 207.

Lawley, P.D. and Brooks, P. (1963) Biochem. J. 89, 127.

Lawley, P.D. and Martin, C.N. (1975) Biochem. J. 145, 85.

Lawley, P.D. and Thacher, C.J. (1970) Biochem. J. 116, 693.

Lepecq, J.B. and Paoletti, C. (1967) J. Mol. Biol. 27, 87.

Lerman, L.S. (1964) J. Cell. Comp. Physiol. 64, Sup. 1, 1.

Lerman, L.S. (1963) Proc. Natl. Acad. Sci. USA 49, 94.

Macgregor, J.T. and Jurd, L. (1978) Mutat. Res. 54, 297.

Main, R.K. and Cole, L.J. (1957) Arch. Biochem. Biophys. 68, 186.

Main, R.K., Wilkins, M.J. and Cole, L.J. (1959a) Science 129, 331.

Main, R.K., Wilkins, M.J. and Cole, L.J. (1959b) J. Amer. Chem. Soc. 81, 6490.

Mardigree, A.A. and Epler, J.L. (1978) Mutat. Res. 58, 231.

Martinson, H.G. (1973a) Biochemistry 12, 139.

Martinson, H.G. (1973b) Biochemistry 12, 2731.

Martinson, H.G. (1973c) Biochemistry 12, 2737.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975) Proc. Natl. Acad. Sci. USA 72, 5135.

McConaughy, B.L., Laird, C.D. and McCarthy, B.J. (1969) Biochemistry 6, 3289.

Mcgrath, R. and Williams, R. (1966) Nature (London) 212, 534.

Mechali, M., deRecondo, A.M. and Girard, M. (1973) Biochem. Biophys. Res. Commun. 54, 1306.

Meneghini, R. (1976) Biochim. Biophys. Acta 425, 419.

Meselson, M.S. and Radding, C.M. (1975) Proc. Natl. Acad. Sci. USA 72, 358.

Middleton, J.H., Edgell, N.H. and Hutchinson, C.A. III (1972) J. Virol. 10, 42.

Miller, J.A. (1970) Cancer Res. 30, 559.

Morino, K., Matsukura, N., Kawachi, T., Ohgaki, H., Sugimora,T. and Hirono, I. (1982) Carcinogenesis 3(1),93.

Muller, W. and Crothers, D.M. (1968) J. Mol. Biol. 35, 251.

Nagao, M., Sugimura, T. and Matsushima, T. (1978) Ann. Rev. Genetics. 12, 117.

Nagao, M., Morita, N., Yahagi, T., Shimizo, M., Kuroyanagi, M., Furuoka, M., Yoshihira, K., Natori, S., Fujino, T. and Sugimura, T. (1981) Environ. Mutagenesis 3, 401.

Newton, B.A. (1967) Biochem. J. 105, (3), 50.

Newton, B.A. and Le Page, R.W.F. (1967) Biochem. J. 105, 50.

O' Brien, R.L., Allison, J.L. and Hahn, F.E. (1966) Biochim. Biophys. Acta 129, 622.

Oka, A., Sugimoto, K. and Takanami, M. (1980) Mol. Gen. Genet. 178, 9.

Oleson, A.E. and Sasakuma, M. (1980) Arch. Biochem. Biophys. 204, 361.

Pakroppa, W., Goebel, W. and Muller, W. (1975) Anal. Biochem. 67, 132.

Pakroppa, W. and Muller, W. (1974) Proc. Natl. Acad. Sci. USA 71 (3), 669.

Pamuckco, A.M., Yalciner, S., Hatcher, J.F. and Bryan, G.T. (1980) Cancer Res. 40, 3468.

Peacocke, A.R. and Skerett, J.N.H. (1956) Trans. Faraday Soc. 52, 261.

Pearson, R.L. and Kelmers, A.D. (1966), J. Biol. Chem. 241, 767.

Poirier, L.A. and Weisberger, E.K. (1979) J. Natl. Cancer. Inst. 62, 833.

Pulkrabeck, P., Leffler, S., Weinstein, I.B. and Grunberger, D. (1977) Biochemistry 16, 3127.

Reinhardt, C.R. and Krugh, T.R. (1978) Biochemistry 17, 4845.

Rushizky, G.W., Shaternikov, V.A., Mezejko, J.H. and Sober, H.A. (1975) Biochemistry 14, 4221.

Sakonju, S., Bogenhagen, D.F. and Brown, D.D. (1980) Cell 19, 13.

Sakore, T.D., Jain, S.C., Tsai, C.C. and Sobell, H.M. (1977) Proc. Natl. Acad. Sci., USA 74, 188.

Scatchard, G. (1949) Ann. N.Y. Acad. Sci., 51, 660.

Schneider, W.C. (1957) in Methods Enzymol (Colowick, S.P. and Kaplan, N.O., eds.) vol.3, p. 680, Academic Press Inc. New York.

Scudiero, D.A. and Strauss, B. (1974) J. Mol. Biol. 83, 17.

Scudiero, D., Henderson, E., Norin, A. and Strauss, B. (1975) Mutat, Res. 29, 473.

Semenza, G. (1957) Ark. Kemi, 11, 89.

Shenk, T.E., Rhodes, C., Rigby, P.W.G. and Berg., P. (1975) Proc. Natl. Acad. Sci., USA 72, 989.

Sheridan, R.B. III and Huang, P.C. (1977) Nucleic Acids Res. 4, 299.

Shishido, K. and Ando, T. (1972) Biochim. Biophys. Acta, 287, 477.

Shishido, K. and Ando, T. (1974) Biochem. Biophys. Res. Commun. 59, 1380.

Shishido, K. and Ando, T. (1975) Agr. Biol. Chem. 39, 673.

Silber, J.R. and Loeb, L.A. (1981) Biochim. Biophys. Acta, 656, 256.

Sinha, M., Goswami, D.N. and Das Gupta, N.N. (1978) Ind. J. Biochem. Biophys. 15 (3) 162.

Sisson, T.R.C. in First International Symposium on Bilirubin Metabolism in the Newborn, Birth Defects, Original Article Series.

Smith, H.O. and Wilcox, K.W. (1970) J. Mol. Biol. 51, 379.

Speck, W.T., Chen, C.C. and Rosenkranz, H.S. (1975) Pediat. Res. 9, 150.

Speck, W.T., Rosenkranz, S. and Rosenkranz, H.S. (1976) Biochim. Biophys. Acta, 435, 39.

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E. and Inouye, M. (1966) Cold Spr. Harb. Symp. Quant. Biol. 31, 77.

Sugimura, T., Nagao, M., Matsushima, T., Yahagi, T., Seino, Y., Shirai, A., Sawamura, M., Natori, S., Yoshihaja, K., Fukuoka, M. and Kuroyanagi, M. (1977) Proc. Jpn. Acad. 5313, 194.

Sutton, W.D. (1971) Biochim. Biophys. Acta 240, 522.

Sutton, W.D. and Walker, P.M.B. (1972) Biochem. J. 128, 193.

Synder, S.L. and Sobocinski, P.Z. (1975) Anal. Biochem. 64, 284.

Tamm, C., Shapiro, S.H., Lipshitz, R. and Chargoff, E. (1953) J. Biol. Chem. 203, 673.

Tiselius, A., Hjerten, S. and Levin, O. (1956) Arch. Biochem. Biophys. 65, 132.

Tsai, C.C., Jain, S.C. and Sobell, H.M. (1977) J. Mol. Biol. 114, 333.

Verly, W.G. (1974) Biochem. Pharmacol. 23, 3.

Verly, W.G. Paquette, Y. and Thibodeau, L. (1973) Nature New Biol. 244, 67.

Vogt, V.M. (1973) Eur. J. Biochem. 33, 192.

Wang, J.C. (1969a) J. Mol. Biol. 43, 25.

Wang, J.C. (1969b) J. Mol. Biol. 43, 263.

Wani, A.A., Hadi, S.M. and Ahmad, N.S. (1978) Experientia, 34, 392.

Wani, A.A. and Hart, R.W. (1981) Biochim. Biophys. Acta, 655, 396.

Waring, M.J. (1970) J. Mol. Biol. 54, 247.

Waring, M.J. (1981) Ann. Rev. Biochem. 50, 159.

Waring, M.J. (1965) J. Mol. Biol. 13, 269.

Warren, E.R., Glaubiger, D. and Kohn, W.K. (1979) Biochim. Biophys. Acta, 562, 41.

Watson, W.A.F. (1982) Mutat. Res. 103, 145.

Webb, R.B. Matina, M.M. and Benson, D.F. (1967) Genetics, 56, 594.

Weigand, R.C., Godson, G.N. and Radding, C.M. (1975) J. Biol. Chem. 250, 8848.

Weinstein, I.B. (1977) Colloq, Int. C.N.R.S. Cancerog, Chimi., 256, 2.

Wells, R.D., Goodman, T.C., Hillen, W., Horn, G.T., Klein, R.D., Larson, J.E., Muller, U.R., Neuendorf, S.K., Panayotatos, N. and Stirdivant, S.M. (1980) Progress in Nucleic Acid. Res. & Mol. Biol. 24, 167.

Wittelberger, S.C. and Hansen, J.N. (1979) Biochim. Biophys. Acta, 565, 125.

Wurst, R.M., Vournakis, J.N. and Maxam, A.M. (1978) Biochemistry, 17, 4493.

Wynder, E.L. and Gori, G.B. (1977) J. Natl. Cancer, Inst., 58, 825.

PUBLICATIONS AND PRESENTATIONS

1 **N.K. ALVI, R.Y. RIZVI & S.M. HADI**

Hydroxyapatite chromatography of DNA with intercalating and non-intercalating agents. Paper presented at the 50th Annual Meeting of the Society of Biological Chemists (India) held at M.S. University of Baroda, 18-20th Nov., 1981.

2 R.Y. RIZVI, N.K. ALVI & S.M. HADI

Effect of alkylation on the secondary structure of DNA. Bioscience Reports, vol. 2, No. 5, 315-322, 1982.

3 N.K. ALVI & S.M. HADI

A sensitive color reaction for the estimation of the trypanocidal drug berenil. Paper presented at the 4th National Congress of Parasitology held at Aligarh Muslim University, Aligarh, 28-30th March, 1982.

4 R.Y. RIZVI, N.K. ALVI & S.M. HADI

Effect of alkylation on the secondary structure of DNA. Paper presented at the 51st Annual Meeting of the Society of Biological Chemists(India) held at Panjab University, Chandigarh, 18-20th Nov., 1982.

5 N.K. ALVI, N.S. AHMAD, SULTAN AHMAD & S.M. HADI

Studies on the interaction of riboflavin with DNA. Paper presented at the 51st Annual Meeting of the Society of Biological Chemists(India) held at Panjab University, Chandigarh, 18-20th Nov., 1982.

6 N.K. ALVI, N.S. AHMAD, SULTAN AHMAD & S.M. HADI

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Effect of alkylation on the secondary structure of DNA

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S_1 nuclease hydrolysis and benzoylated naphthoylated DEAE-cellulose (BND-cellulose) chromatography have been used to demonstrate that alkylation of DNA by dimethyl sulfate at neutral pH leads to the production of partially denatured molecules under conditions where no significant depurination occurs. DNA was alkylated with increasing concentrations of the alkylating agent, and subjected to enzymatic degradation and binding to BND cellulose. An increasing degree of DNA hydrolysis and adherence to BND cellulose was seen. On hydroxyapatite chromatography the alkylated DNA still eluted at the position of double-stranded molecules suggesting the presence of partially denatured regions. The presence of salt had a preventive effect on such denaturation.

Many anticancer drugs used in clinical and experimental work are DNA-alkylating agents. Under appropriate circumstances these agents are themselves capable of producing cancer (Waring, 1981; Hsiung et al., 1976). Lawley and co-workers have shown that the main alkylation sites on double-stranded DNA are the N-7 of guanine and N-3 of adenine (Lawley & Brooks, 1963; Lawley & Martin, 1975). Alkylated purines are lost by spontaneous hydrolysis of the glycosidic linkage, a process known as depurination (Verly, 1974). At alkaline pH, the apurinic site is converted into a single-strand break by hydrolysis of a neighbouring phosphodiester bond (Tamm et al., 1953). Using hydroxyapatite chromatography we have earlier shown that alkylation of DNA by dimethyl sulfate at neutral pH may lead to denaturation of molecules under conditions where no significant depurination occurs (Wani et al., 1978). However, the presence of partially denatured DNA molecules, as possible intermediates, could not be detected by this technique. It is considered likely that excision repair of chemically induced DNA damage occurs by two separate pathways (Peter Karran et al., 1977): (a) the base excision (apurinic) repair pathway, in which reacted DNA depurinates spontaneously or via the action of an N-glycosidase; (b) the nucleotide excision pathway in which an endonuclease specific for thymine dimers, for chemical adducts, or for regions of local denaturation is followed by the action of exonuclease, polymerase, and ligase activities. In the present report we have attempted to demonstrate the formation of partially denatured regions in alkylated DNA by the use of S_1 nuclease hydrolysis and benzoylated naphthoylated DEAE-cellulose (BND-cellulose) chromatography.

Materials and Methods

Calf-thymus deoxyribonucleic acid (sodium salt, average mol. wt., one million), BND-cellulose, and caffeine were obtained from Sigma Chemical Company. The DNA was used without further purification. S_1 nuclease was purchased from Boehringer. Dimethyl sulfate was from May and Baker Ltd., England. Hydroxyapatite was prepared as described by Bernardi (1971).

A 1-2 mg/ml solution of DNA in TNE (0.01 M Tris-HCl, pH 7.5, 0.01 M, or 0.1 M NaCl and 2 x 10^{-4} MEDTA), was methylated by adding sufficient dimethyl sulfate (DMS) to obtain the desired DNA nucleotide/DMS molar ratio. The solution was gently shaken at 25° for 1 h. The acid released by the hydrolysis of DMS was neutralized by the addition of enough 0.1 M NaOH, and the pH was maintained between 6 and 7. BND-cellulose chromatography was carried out as described by Peter Karran et al. (1977). Double-stranded DNA elutes from BND-cellulose with 1 M NET (1 M NaCl - 10^{-4} M EDTA - 0.01 M Tris-HCl, pH 7.5). DNA with single-stranded regions requires 50% formamide in 1 M NET or 2.0% caffeine in 1 M NET. Hydroxyapatite chromatography was done according to Bernardi (1971). DNA was measured in column fractions by the procedure of Burton (1956). The melting profile of DNA using S_1 nuclease was determined as described by Case and Baker (1975).

Results and Discussion

Degradation of alkylated DNA by S_1 nuclease

DNA was alkylated with an increasing molar ratio of dimethyl sulfate and subjected to hydrolysis by the single-strand-specific nuclease S_1. The results given in Fig. 1 show that with increasing enzyme concentration, production of maximum acid-soluble material increased with DMS molar ratio used for alkylation. Controls using native and denatured DNA showed 4% and 100% degradation, respectively. Thus alkylation transforms DNA into an effective substrate for S_1 nuclease, possibly due to the formation of partially denatured regions in the native molecule. As already mentioned, alkylation of DNA may lead to denaturation even in the absence of significant depurination. This was considered to be due to a destabilization of the secondary structure caused by an accumulation of positive charges on opposite strands (Wani et al., 1978).

S_1 nuclease has been utilized in studying the secondary structure of DNA through a variety of approaches; e.g., it has been used to determine the thermal melting profile through digestion of the denatured DNA strands. Fig. 2 shows such an experiment with calf-thymus DNA alkylated at a DNA nucleotide/DMS molar ratio of 1:1. The mid-range melting temperature (Tm) of control native DNA was determined to be 81°C. The melting profile of alkylated DNA is somewhat unusual since even at temperatures below 60°C considerable hydrolysis of DNA is observed. This is presumably because of depurination of alkylated bases at these temperatures (Verly et al., 1973). This would result in partial denaturation of the double-stranded molecule, rendering these regions digestible by S_1 nuclease. Also, complete hydrolysis of alkylated DNA is not observed, even at 95°C.

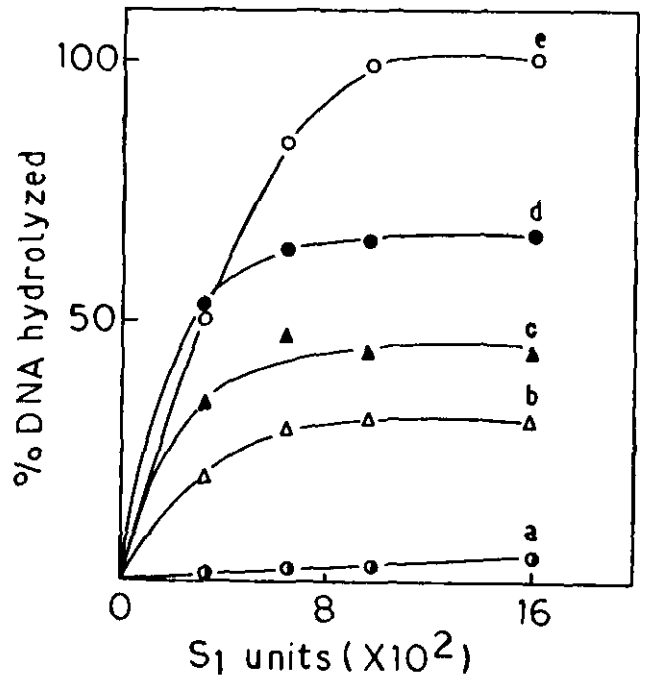

Fig. 1. Degradation of alkylated DNA by S_1 nuclease at various DNA nucleotide/DMS molar ratios. DNA was alkylated as described in the text, and subsequently dialysed against 50 vol. of TNE containing 0.1 M NaCl. The reaction mixture in 1 ml contained 500 µg native, alkylated, or denatured DNA, 0.1 M acetate buffer, pH 4.5, containing 1 mM $ZnSO_4$, and enzyme units as indicated. The incubation was at 40° for 2 h and the reaction stopped by the addition of 0.2 ml of 10 mg/ml bovine serum albumin and 1.0 ml cold 14% PCA. Acid-soluble nucleotides were measured by the diphenylamine procedure (Schneider, 1957). (a) Native DNA; (b,c,d) DNA alkylated with DNA nucleotide/DMS molar ratio of 1:1, 1:2, and 1:4, respectively; (e) denatured DNA.

This may be due to the formation of a limited number of interstrand crosslinks originating from the exposed aldehyde function at the apurininc sites (Burnotte & Verly, 1972). At higher DMS molar ratios progressively decreasing hydrolysis at 95°C was seen, thereby supporting the above idea.

BND-cellulose chromatography of alkylated DNA

BND-cellulose has been used to measure growing points in replicating DNA (Scudiero & Strauss, 1974) and to detect interruptions in human cellular DNA treated with methylmethane sulfonate, an

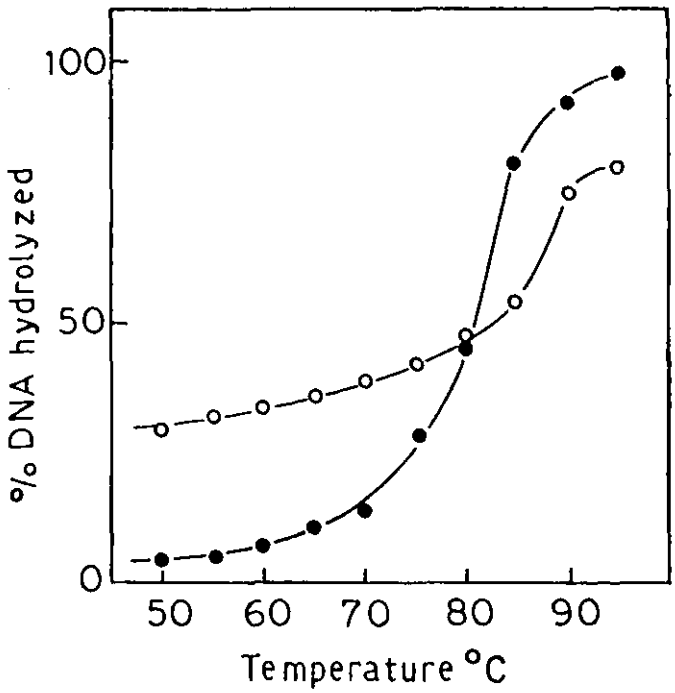

Fig. 2. Thermal denaturation of native and alkylated DNA as measured by the degree of S_1 nuclease digestion. DNA was alkylated as described in the text and dialysed against 0.1 M NaCl TNE. Samples containing 300 µg DNA were heated to the desired temperature for 8 min and then quickly quenched by the addition of 2 vol. of ice-cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 600 units of S_1 nuclease at 40° for 2 h. The reaction was terminated and processed as described in Fig. 1. Native DNA (●-●); alkylated DNA, nucleotide/DMS ratio 1:1 (O-O).

alkylating agent (Peter Karran et al., 1977). Adherence of DNA to BND-cellulose, represented by the fraction which elutes with 2.0% caffeine in 1 M NET, is considered to be due to two properties: binding of single-stranded regions, or binding of aromatic residues to the resin (Peter Karran et al., 1977). In Fig. 3b,c,d, and e is shown the BND-cellulose chromatography of DNA samples alkylated with increasing molar ratios of DMS in 0.1 M NaCl TNE. DNA without single-stranded breaks or regions, obtained by pre-chromatography of calf-thymus DNA on BND-cellulose, was used in these experiments. Increasing numbers of DNA molecules show adherence to BND-cellulose with a concomitant decrease in the material eluting with 1 M NET. The DNA sample alkylated at a DNA nucleotide/DMS molar ratio of 1:6, where almost 80% molecules adhere to BND cellulose, was also

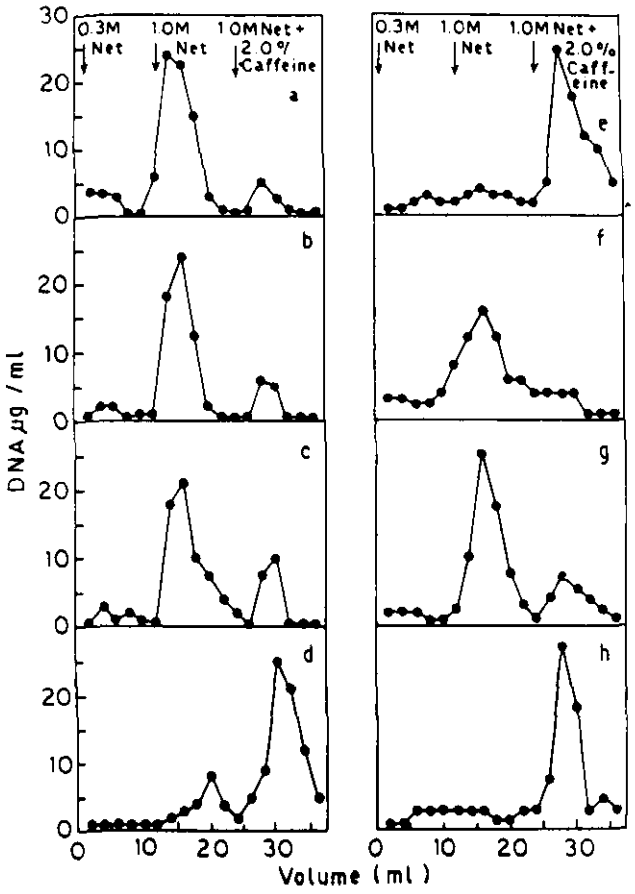

Fig. 3. Chromatography of alkylated DNA on BND-cellulose under various conditions. To obtain DNA without single-stranded breaks or regions, calf-thymus DNA was prechromatographed on BND-cellulose. The 1 M NET eluate was dialysed against 0.01 M or 0.1 M NaCl TNE. Control DNA was also dialysed. 100-µg DNA samples were alkylated for 1 h with sufficient dimethyl sulfate (diluted 10-fold in TNE) to obtain the desired molar ratio. The alkylated DNA was directly applied to the column (1 x 2.5 cm) and step-eluted with 0.3 M NET, 1 M NET, and 1 M NET plus 2.0% caffeine. 2-ml fractions were collected at the rate of 8 ml/h. DNA was measured by the diphenylamine method of Burton (1956). Recoveries in all experiments ranged between 80-100%. (a) Control; (b,c,d,e) DNA alkylated in 0.1 M NaCl with a DNA nucleotide/DMS molar ratio of 1:2, 1:4, 1:6, and 1:8, respectively; (f) same as d but treated with S_1 nuclease after alkylation; (g) control DNA in 0.01 M NaCl TNE; (h) DNA in 0.01 M NaCl TNE alkylated with a nucleotide/DMS ratio of 1:2.

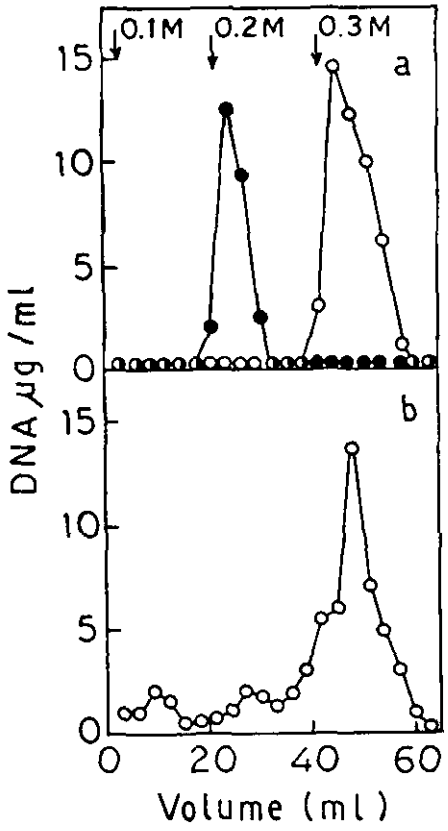

Fig. 4. Hydroxyapatite chromatography of DNA alkylated with a nucleotide/DMS molar ratio of 1:6 in 0.1 M NaCl TNE. DNA without single-stranded regions was obtained by pre-chromatography on BND-cellulose, and a 200-µg sample was alkylated as described in the text. It was loaded on a 1 x 3 cm column, previously equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, and immediately followed by elution with sodium phosphate buffer (pH 7.0) of indicated molarities. 3-ml fractions were collected at the rate of 10 ml/h. Recovery was approximately 78%. (a) Native DNA (○-○), denatured DNA (●-●); (b) alkylated DNA.

chromatographed on hydroxyapatite (Fig. 4). Almost all the DNA eluted at the position of double-stranded DNA suggesting that adherence to BND-cellulose is not the result of complete denaturation of DNA molecules. Alkaline hydrolysis of alkylated DNA samples used in these experiments showed no production of acid-soluble nucleotides, suggesting that no significant depurination of alkylated sites had occurred. In Fig. 3f the DNA was alkylated at a molar ratio of 1:6, and subsequently treated with S_1 nuclease before applying to BND-cellulose. As expected, the remaining DNA after digestion with the

nuclease does not adhere to the resin, and elutes with 1 M NET. It is therefore concluded that alkylation by DMS under our conditions causes the formation of partially denatured DNA molecules. When DNA was alkylated in 0.01 M NaCl TNE at a DNA nucleotide/DMS molar ratio of 1:2, 78% of the molecules adhere to BND-cellulose (Fig. 3h) compared with 18% in the presence of 0.1 M NaCl TNE (Fig. 3b). A control DNA sample kept in 0.01 M NaCl TNE showed only 25% adherence.

Monofunctional alkylating agents alkylate DNA at two sites: bases and phosphates. However, the major sites of alkylation with DMS are the N-7 position of guanine and N-3 position of adenine, and it causes minimum alkylation of DNA phosphates (Lawley & Thacher, 1970). Our results suggest that alklyation of DNA may lead to the formation of partially denatured molecules even in the absence of depurination of alkylated bases. A possible mechanism could be that the positive charges of the quaternized alkylated bases through repulsion may adversely affect the forces stabilizing the secondary structure. This is strengthened by the observation that the presence of salt has a preventive effect on the formation of partially denatured molecules. Hsiung et al. (1976) have postulated that partial denaturation of alkylated DNA at higher pH values may occur through the disruption of hydrogen bonding due to a base-catalyzed imidazole ring opening of the quaternized N-7 guanine. There is now considerable evidence that changes in DNA conformation, such as distortions in the secondary structure produced by intercalating agents or regions of local denaturation, are recognized by DNA repair or other nucleases producing strand breakage (Duncan et al., 1976; Warren et al., 1979). Indeed, it has been suggested that the cytotoxic effect of many drugs may be better related to DNA damage as a result of the action of nucleases that recognize distortion of the helical structure, rather than to the physical presence of the bound drug itself (Waring, 1981).

References

Bernardi G (1971) in *Methods Enzymol.* (Colowick SP & Kaplan NO, eds), vol **21**, pp 95-139, Academic Press, New York.

Burnotte J & Verly WG (1972) Biochim. Biophys. Acta **262**, 449-452.

Burton K (1956) Biochem. J. **62**, 315-323.

Case TS & Baker FR (1975) Anal. Biochem. **64**, 477-484.

Duncan J, Hamilton L & Friedberg E (1976) J. Virol. **19**, 338-345.

Hsiung JH, Lown W & Johnson D (1976) Can. J. Biochem. **54**, 1047-1053.

Lawley PD & Brooks P (1963) Biochem. J. **89** 127-138.

Lawley PD & Martin CN (1975) Biochem. J. **145**, 85-91.

Lawley PD & Thacher CJ (1970) Biochem. J. **116**, 693-707.

Peter Karran N, Higgins P & Strauss B (1977) Biochemistry **16**, 4483-4490.

Schneider WC (1957) in *Methods Enzymol.* (Colowick SP & Kaplan NO, eds), vol **3**, pp 680-684, Academic Press, New York.

Scudiero DA & Strauss B (1974) J. Mol. Biol. **83**, 17-34.
Tamm C, Shapiro SH, Lipshitz R & Chargoff E (1953) J. Biol. Chem. **203**, 673-688.
Verly WG (1974) Biochem. Pharmacol. **23**, 3-8.
Verly WG, Paquette Y & Thibodeau L (1973) Nature New Biol. **244**, 67-69.
Wani AA, Hadi SM & Ahmad NS (1978) Experientia **34**, 392-394.
Waring JM (1981) Ann. Rev. Biochem. **50**, 159-192.
Warren ER, Glaubiger D & Kohn WK (1979) Biochim. Biophys. Acta **562**, 41-50.